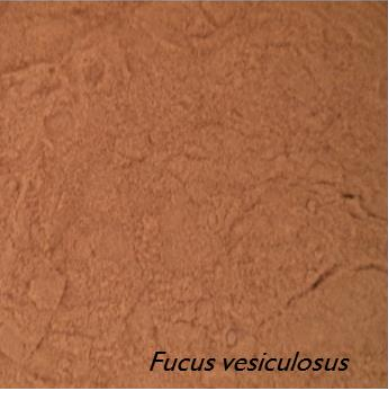
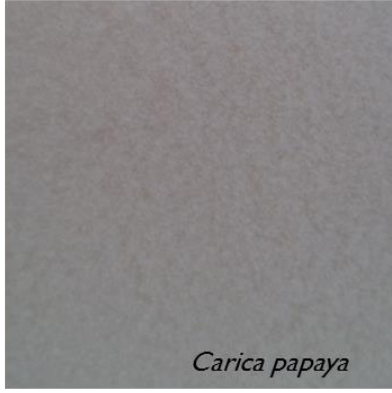
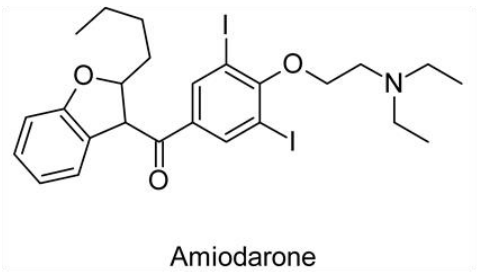
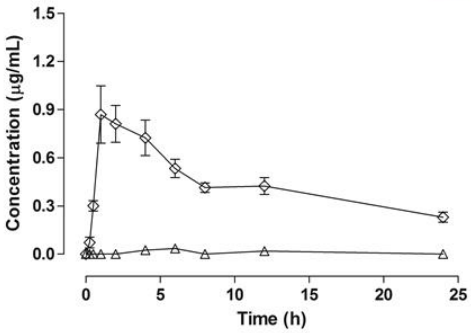






Amiodarone



Márcio José de Abreu Marques Rodrigues

EVALUATION OF THE EFFECT OF HERBAL EXTRACTS USED IN SLIMMING REGIMENS IN THE KINETIC PROFILE OF NARROW THERAPEUTIC RANGE DRUGS USED FOR CARDIOVASCULAR PATHOLOGIES: THE AMIODARONE

Tese de Doutoramento em Ciências Farmacêuticas, especialidade de Farmacologia e Farmacoterapia, orientada pelo Professor Doutor Amílcar Falcão e pelo Professor Doutor Gilberto Alves e apresentada à Faculdade de Farmácia da Universidade de Coimbra

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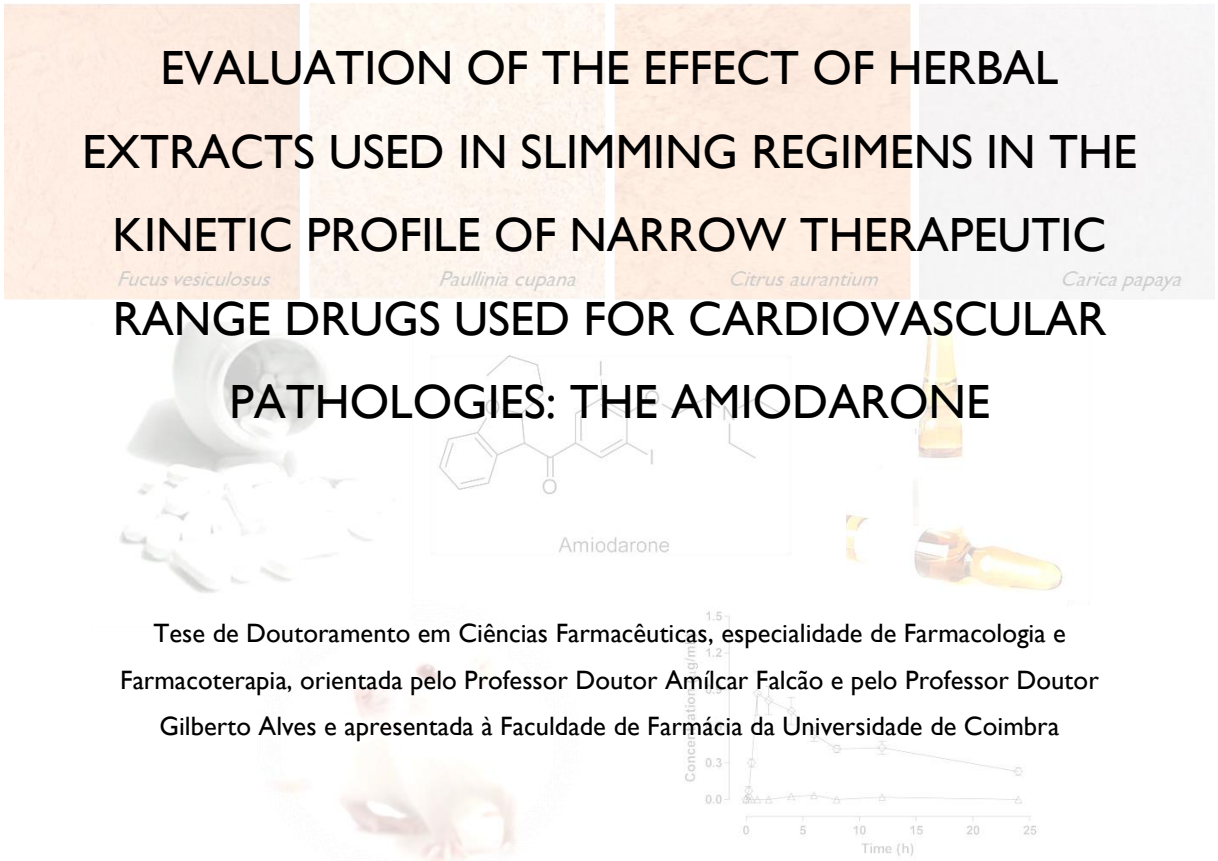

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The research work presented in this thesis was performed in strict collaboration of the Laboratory of Pharmacology, Faculty of Pharmacy, University of Coimbra and the Health Sciences Research Centre, Faculty of Health Sciences, University of Beira Interior, under the supervision of Professor Amílcar Falcão and Professor Gilberto Alves and it was supported by Portuguese Foundation for Science and Technology (SFRH/BD/61901/2009).

Os trabalhos experimentais apresentados nesta tese foram realizados em estreita colaboração entre o Laboratório de Farmacologia da Faculdade de Farmácia da Universidade de Coimbra e o Centro de Investigação em Ciências da Sáude da Faculdade de Ciências da Saúde da Universidade da Beira Interior, sob a supervisão do Professor Doutor Amílcar Falcão e do Professor Doutor Gilberto Alves, e apoiados pela Fundação para a Ciência e a Tecnologia (SFRH/BD/61901/2009).

Aos meus pais

Ao meu irmão

E à minha irmã

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ABBREVIATIONS

ABBREVIATIONS

ABC	Adenosine 5'-triphosphate binding cassette
AM	Amiodarone
AOPP	Advanced oxidative protein product
ATP	Adenosine 5'-triphosphate
AUC	Area under concentration-time curve
AUC_{0-t}	AUC from time zero to the last sampling time
$AUC_{0-\infty}$	AUC from time zero to infinite
BLQ	Below the limit of quantification
C_{last}	Last quantifiable concentration
C_{max}	Peak concentration
CPL	*Carica papaya* lipase
CYP	Cytochrome P450
DAD	Diode-array detection
DDEA	Di-*N*-desethylamiodarone
DSHEA	Dietary Supplement Health and Education Act
EMA	European Medicines Agency
FDA	Food and Drug Administration
HMPC	Herbal Medicinal Products Committee
HPLC	High performance liquid chromatography
INR	International normalized ratio
i.p.	Intraperitoneal
IS	Internal standard
k_{el}	Apparent terminal elimination rate constant
LC-MS	Liquid chromatography-mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
LLE	Liquid-liquid extraction
MDR	Multi-drug resistance
MEPS	Microextraction by packed sorbent
mRNA	Messenger ribonucleic acid
MRP2	Multi-drug resistance-associated protein-2
MRT	Mean residence time
OATP-B	Organic anion transporting polypeptide B

OATP1B1	Organic anion transporting polypeptide 1b1
3'OH-MDEA	*n*-3-hydroxybutyl mono-*N*-desethylamiodarone
P-gp	P-glycoprotein
PP	Protein precipitation
QC	Quality control
r^2	Regression coefficient
SD	Standard deviation
SEM	Standard error of the mean
SPE	Solid-phase extraction
TAM	Tamoxifen
TDM	Therapeutic drug monitoring
t_{max}	Time to reach peak concentration
$t_{1/2el}$	Apparent terminal elimination half-life
$t_{1/2alpha}$	Alpha-phase distribution half-life
$t_{1/2beta}$	Beta-phase elimination half-life
UGT	Uridine 5'diphospho-glucuronyltransferase
V_d	Apparent volume of distribution

PUBLICATIONS

PUBLICATIONS

RODRIGUES, M.; ALVES, G.; FERREIRA, A.; QUEIROZ, J.; FALCÃO, A. – A rapid HPLC method for the simultaneous determination of amiodarone and its major metabolite in rat plasma and tissues: a useful tool for pharmacokinetic studies. **J. Chromatogr. Sci.** 51:4 (2013) 361-370.

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RODRIGUES, M.; ALVES, G.; ROCHA, M.; QUEIROZ, J.; FALCÃO, A. – First liquid chromatographic method for the simultaneous determination of amiodarone and desethylamiodarone in human plasma using microextraction by packed sorbent (MEPS) as sample preparation procedure. **J. Chromatogr. B** 913-914 (2013): 90-97.

RODRIGUES, M.; ALVES, G.; LOURENÇO, N.; FALCÃO, A. – Herb-drug interaction of *Paullinia cupana* (Guarana) seed extract on the pharmacokinetics of amiodarone in rats. **Evid. Based Complement. Alternat. Med.** 2012 doi:10.1155/2012/428560.

RODRIGUES, M.; ALVES, G.; FALCÃO, A. – Investigating herb-drug interactions: the effect of *Citrus aurantium* fruit extract on the pharmacokinetics of amiodarone in rats. **Food Chem. Toxicol.** 60 (2013) 153-159.

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ABSTRACT/RESUMO

ABSTRACT

The use of plants for the prevention and treatment of various health diseases has been a practice that comes from the ancient civilizations. However, in recent years has been observed an increase in the use of herbal products. In developed countries is particularly worrying the increasing consumption of herbal weight loss medicines/dietary supplements as alternative or complement to the traditional programs of body weight reduction. In addition, this issue is even more serious because among the general population prevails the notion that herbal preparations are devoid of health risks due to the natural origin of the constituents. Indeed, only a minority recognizes that herbal medicinal products are complex mixtures of phytochemicals, which may be substrates, inhibitors or inducers of the same biological systems that are involved in the biodisposition of conventional drugs, existing, therefore, a high potential for clinically significant pharmacokinetic interactions. Accordingly, considering the close relationship between obesity and cardiovascular diseases, as well as the scarcity of scientific information available about the safety of herbal slimming medicinal products, it is absolutely justified to assess the potential for pharmacokinetic interactions between the most relevant herbal weight loss extracts and high-risk drugs required in multiple cardiovascular diseases such as the case of amiodarone (AM). Thus, from the perspective of protecting public health, it is warranted to characterize the interference of standardized herbal extracts commonly used in weight loss programs *(e.g. Fucus vesiculosus, Paullinia cupana, Citrus aurantium* and *Carica papaya)* on the pharmacokinetic profile of AM.

This thesis emerged in this context and aimed to carry out the *in vivo* nonclinical systematic assessment of the potential for occurrence of significant herb-drug interactions between commonly used standardized herbal weight loss extracts and AM, a drug of narrow safety range widely used in clinical practice for the management of cardiovascular diseases. One technique of high performance liquid chromatography (HPLC) with diode-array detection (DAD) was developed and fully validated for adequate quantification of AM and its main metabolite (mono-*N*-desethylamiodarone) in human plasma in order to provide a useful analytical tool for clinical purposes. A second technique was developed and validated in plasma and heart, liver, kidney, and lung tissue homogenates of rats – species used for *in vivo* investigations.

The herbal extracts that have more pronounced effects on the pharmacokinetics of AM were *F. vesiculosus*, *P. cupana* and *C. papaya* extracts. *F. vesiculosus* extract and *P.*

cupana extract following the co-administration with AM showed a significant reduction of the peak concentration of AM as well as a reduction in the extent of systemic exposure to AM. For the first time were reported herb-drug interactions between *F. vesiculosus* extract and *P. cupana* extract with AM, which determined a considerable decrease on AM bioavailability in rats. Therefore, the therapeutic efficacy of AM may be compromised by the concurrent administration of herbal slimming medicines/dietary supplements containing *F. vesiculosus* or *P. cupana.*

On the other hand, it should be highlighted that the pre-treatment with *C. aurantium* extract significantly increased the peak concentration of AM, while the extension of systemic exposure was comparable between both groups. At last, in the rats pre-treated with *C. papaya* extract was observed a significant increase in the systemic exposure of AM.

In conclusion, the findings reported along this thesis showed the relevance of screening herb-drug pharmacokinetic interactions. The knowledge of relevant herb-drug interactions is certainly useful to help in the preparation of appropriate herbal monographs such those in course by the Herbal Medicinal Products Committee (HMPC) of the European Medicines Agency (EMA). The availability of this kind of information gathered together in a single and easily accessible document (monograph) may be of great value to promote a more rational and safer use of herbal products, having high importance in terms of protecting the public health.

RESUMO

O uso de plantas para a prevenção e tratamento de várias doenças tem sido uma prática desde as civilizações antigas. No entanto, nos últimos anos tem-se observado um aumento da utilização de produtos à base de plantas. Nos países desenvolvidos tem sido particularmente preocupante o aumento do consumo de produtos de emagrecimento/suplementos alimentares como alternativa ou complemento aos programas tradicionais de perda de peso corporal. Além disso, esta questão é ainda mais grave porque entre a população prevalece, em geral, a noção de que as preparações à base de plantas são desprovidas de riscos para a saúde devido à origem natural dos constituintes. De facto, apenas uma minoria reconhece que os produtos medicinais à base de plantas são misturas complexas de fitoquímicos, que podem ser substratos, inibidores ou indutores dos mesmos sistemas biológicos que estão envolvidos na biodisposição de fármacos convencionais, existindo, por isso, um elevado potential para interações farmacocinéticas significativas. Por conseguinte, considerando a relação estreita entre a obesidade e as doenças cardiovasculares, bem como a escassez de informação científica disponível sobre a segurança de produtos medicinais de emagrecimento à base de plantas, é absolutamente justificado avaliar-se o potencial de interação de base farmacocinética entre os extratos de emagrecimento de origem vegetal mais relevantes e fármacos de maior risco usados em várias doenças cardiovasculares, como é o caso da amiodarona (AM). Assim, tendo em perspetiva a proteção da saúde pública, justifca-se a caraterização da interferência de extratos padronizados de plantas medicinais comummente utilizadas em programas de perda de peso (por exemplo, *Fucus vesiculosus*, *Paullinia cupana*, *Citrus aurantium* e *Carica papaya*) no perfil farmacocinético da AM.

Esta tese surgiu neste contexto e visou conduzir estudos não-clínicos *in vivo* para a avaliação do potencial de ocorrência de interações significativas extrato-fármaco entre os extratos padronizados para perda de peso e a AM, um fármaco de margem terapêutica estreita amplamente utilizada na prática clínica para o tratamento de doenças cardiovasculares. Para tornar estes estudos exequíveis, foi desenvolvida e validada uma técnica de cromatografia líquida (HPLC) com um detetor de fotodíodos (DAD) para a quantificação adequada da AM e do seu principal metabolito (mono-*N*-desetilamiodarona) em plasma humano, a fim de disponibilizar uma ferramenta analítica para aplicação clínica. A

segunda técnica foi desenvolvida e validada em plasma e em homogeneizados de tecido do coração, fígado, rim e pulmão de ratos – espécie utilizada nos ensaios *in vivo*.

Os extratos vegetais testados que mostraram efeitos mais pronunciados na farmacocinética da AM foram o *F. vesiculosus*, a *P. cupana* e a *C. papaya*. Os extratos de *F. vesiculosus* e *P. cupana* após a coadministração com a AM mostraram uma redução significativa da concentração máxima de AM bem como uma redução na extensão de exposição sistémica à AM. Pela primeira vez foram descritas interações extrato-fármaco entre o extrato de *F. vesiculosus* e de *P. cupana* com a AM, que determinaram uma diminuição considerável na biodisponibilidade da AM em ratos. Portanto, a eficácia terapêutica da AM pode ser comprometida pela administração concomitante de extratos medicinais de emagrecimento/suplementos dietéticos que contêm *F. vesiculosus* ou *P. cupana*.

Por outro lado, deve ser realçado que o pré-tratamento com extrato de *C. aurantium* aumentou significativamente o pico de concentração plasmática (C_{max}) da AM, enquanto que a extensão de exposição sistémica foi comparável entre ambos os grupos. Por último, nos ratos pré-tratados com o extrato de *C. papaya* foi observado um aumento significativo da exposição sistémica à AM.

Em conclusão, os resultados descritos ao longo desta tese mostram a relevância da investigação de interações farmacocinéticas extrato-fármaco. O conhecimento das interações relevantes extrato-fármaco é certamente útil para ajudar na elaboração de monografias apropriadas, tais como aquelas em curso pelo Comité dos Produtos Medicinais à Base de Plantas (HMPC) da Agência Europeia do medicamento (EMA). A disponibilidade deste tipo de informação reunida num documento único e de fácil acesso (monografia) pode ser de grande valia para promover uma utilização mais racional e segura dos produtos à base de plantas, tendo grande importância em termos de proteção da saúde pública.

CHAPTER I

GENERAL INTRODUCTION

I. GENERAL INTRODUCTION

I.1. INTRODUCTION

The use of plants, parts of plants and isolated phytochemicals for the prevention and treatment of various health diseases has been a practice that comes from the ancient civilizations (SAHOO, MANCHIKANTI and DEY, 2010). However, in recent years has been observed an increase in the use of herbal products (BENT, 2008; PHUA; ZOSEL and HEARD, 2009). A survey of the World Health Organization indicated that about 70-80% of the world populations rely on non-conventional medicines mainly of herbal sources in their primary health care (DEHGHANI *et al.,* 2012). In fact, many patients worldwide have extensively used herbal products concomitantly with prescribed drugs. Some estimates indicate that 40-60% of the patients do not inform the health care providers about the concomitant use of herbal medicinal products with prescribed drugs (TARIRAI, VILJOEN and HAMMAN, 2010).

There is a general belief that herbal medicines are safe because of the natural origin of its constituents. However, herbal medicines are not free of adverse effects and are not devoid of risks (e.g. herb-drug interactions) (HERMANN and VON RICHTER, 2012). On the contrary, considering that more people use herbal medicines to promote health or to control various chronic diseases, for which they are often taking concomitantly multiple prescribed or over-the-counter drugs, there is an increased possibility for pharmacokinetic and/or pharmacodynamic interactions (VIEIRA and HUANG, 2012).

The use of herbal products is also complicated by other problems such as the lack of legislation imposing stricter control of herbal medicinal products and the lack of adequate quality control. Due to these reasons, the herbal products are frequently contaminated with heavy metals and adulterated with pharmaceuticals, and prohibited animal and plant ingredients. These contaminants could put unknowing consumers at risk of adverse side effects and drug interactions (TACHJIAN, MARIA and JAHANGIR, 2010). The lack of strict guidelines on the assessment of safety and efficacy, quality control, safety monitoring and knowledge of herbal medicines are the main shortcomings found in different regulatory systems and that should be improved in the future.

I.2. REGULATION OF HERBAL MEDICINES

Despite its widespread use, there is still limited data on safety and efficacy of the herbal products currently on the market because herbal dietary supplements/medicines are mostly viewed as food supplements and not as drugs. In the United States they are not strictly regulated as drugs by the Food and Drug Administration (FDA). Instead, under the Dietary Supplement Health and Education Act (DSHEA), the dietary supplements can be marketed without evidence to support efficacy and safety of active ingredients and without regarding accuracy of active ingredients. If a dietary supplement appears to be unsafe after being marketed, the FDA can then decide to remove or not the product from the market (EGRAS *et al.,* 2011).

In the European Union the herbal medicinal products are regulated through the European Directive (2004/24/EC), which was prepared aiming at protecting public health and at the same time secure the free movement of herbal medicinal products within the Community. Although most of the individual herbal medicinal products are licensed nationally in each of the Member States, the process for licensing, the information required on herbal substances and the manufacturing process will be increasingly harmonized across the European Union.

The European Directive (2001/83/EC) requires that an application for authorization to place an herbal medicinal product on the market has to be accompanied by a dossier containing results of pre-clinical and/or clinical trials carried out on the product and thus providing its quality, safety and efficacy. These results will not be necessary if the applicant can demonstrate by detailed references to published scientific literature that the constituent or the constituents of the medicinal product has or have a well-established medicinal use with recognized efficacy and an acceptable level of safety. However, even a long traditional use does not exclude all the concerns about product's safety and, therefore, the competent authorities should be entitled to ask for all data necessary for assessing their safety. This is the so-called simplified registration procedure for certain traditional herbal medicinal products that presents particular characteristics especially the long use in folk medicine. In addition, the European Directive (2004/24/EC) also introduces a new subcategory of herbal medicinal products, the so called *traditional herbal medicinal products*, in which the safety needs to be shown while efficacy does not and only the traditional indications in specified conditions must be plausible.

The new classification of herbal medicinal products will require a change of regulatory competences, both at European and National level. Herbal medicines, previously considered as food supplements and regulated as food, will be now considered as medicinal products. Therefore, this implies a passage of jurisdiction firstly from the European food departments, first to the European Medicines Agency (EMA) and successively to the national regulatory agencies of drugs of Member States (CALAPAI, 2008).

A new committee, the Herbal Medicinal Products Committee (HMPC), has been formed inside EMA with responsibility for herbal medicinal products. One of the objectives of the HMPC is to prepare a list of herbal substances, preparations and combinations for use in traditional herbal medicinal products. Thus, the EMA established an action Plan for Herbal Medicines 2010-2011 in which one of the objectives was to improve the output of the Committee on Herbal Medicinal Products, in particular by increasing the quality and number of Monographs and List entries. In each herbal monograph are defined the clinical indications, posology, method of administration, contraindications, precautions use, interactions, data on pregnancy, and other aspects (CALAPAI, 2008). This call for Monographs could be related to the widespread use of herbal products and probably to the increased risk of potential herb-drug interactions.

In Portugal, the herbal medicines could be included in different legislation, according to their characteristics; the Decree-Law (136/2003, of June 28) includes the dietary supplements, which are under the jurisdiction of the Ministry of Agriculture, and the Decree-Law (176/2006, of August 30) contemplates the medicines. The legislation of the Decree-Law (176/2006, of August 30) referring to the Medicines Code transposes the European Directive (2004/24/EC).

I.3. HERB-DRUG INTERACTIONS

The interactions of herbal medicines with drugs are a major safety concern, especially for drugs with a narrow therapeutic range [e.g. warfarin, digoxin and amiodarone (AM)]. As consequence potentially severe and perhaps even life-threatening adverse effects may occur because the pharmacokinetics and/or pharmacodynamics of the drug may be altered with clinical significance by the combination with herbal medicines (ZHOU *et al.,* 2007).

Furthermore, herbal medicines are particularly prevalent among older people. The elderly are more likely to receive several drugs together and are also more sensitive to chemicals. In addition, it must be borne in mind that herbal products are sometimes intended to be taken over a long period of time, which can imply an increased possibility for enzyme inhibition or induction and to potentiate other mechanisms of herb-drug interactions.

A great public health concern and awareness about the subject of herb-drug interactions arose due to the discovery that St. John's wort (*Hypericum perforatum)* was implicated in significant and unequivocal herb-drug interactions (PISCITELLI *et al.,* 2000; MANNEL, 2004; CHAVEZ, JORDAN and CHAVEZ, 2006; MARKOWITZ and ZHU, 2012). This discovery could represent the "tip of the iceberg" of the literally thousands of available dietary supplements, many of which can similarly interact with concomitant medications. Consequently, some efforts to screen numerous botanical products are in progress given the widespread use of these herbal products among the general population (MARKOWITZ, VON MOLTKE and DONOVAN, 2008). In these studies are generally involved the more commonly used products and its influence on major metabolic enzymes has been investigated (MARKOWITZ and ZHU, 2012).

I.3.1 MECHANISMS OF HERB-DRUG INTERACTIONS

The phytochemicals are handled in the body through the same type of processes involved in drug biodisposition (VENKATARAMANAN, KOMOROSKI and STROM, 2006). Consequently, herb-drug interactions may occur through the same pharmacokinetic and pharmacodynamic mechanisms underlying drug-drug interactions (ZHOU *et al.,* 2007; IZZO, 2012).

The pharmacodynamic herb-drug interactions have been less studied than pharmacokinetic-based herb-drug interactions, but the implications of pharmacodynamic interactions are not less important in terms of changes in pharmacological response. Pharmacodynamic herb-drug interactions may involve changes in the pharmacological effects of the drug through additive or synergistic actions, i.e. potentiating the pharmacological/toxicological action of drugs, or antagonistic actions, i.e. the herbal medicines reduce the activity of drugs (TARIRAI, VILJOEN and HAMMAN, 2010; IZZO, 2012). For example, *H. perforatum* when taken with antidepressants belonging to the class of selective serotonin reuptake inhibitors can lead to a pharmacodynamic interaction known as the serotonin syndrome that is characterize by agitation, hyperthermia, diaphoresis, tachycardia, and muscular disturbances, which occurs as the result of high levels of serotonin in the brain (VENTOLA, 2010).

Taking into account the principal objectives of the present thesis a main focus was given to the pharmacokinetic-based herb-drug interactions. The pharmacokinetic herb-drug interactions can occur as a result of changes in absorption, distribution, metabolism and excretion of drugs (Figure I.1).

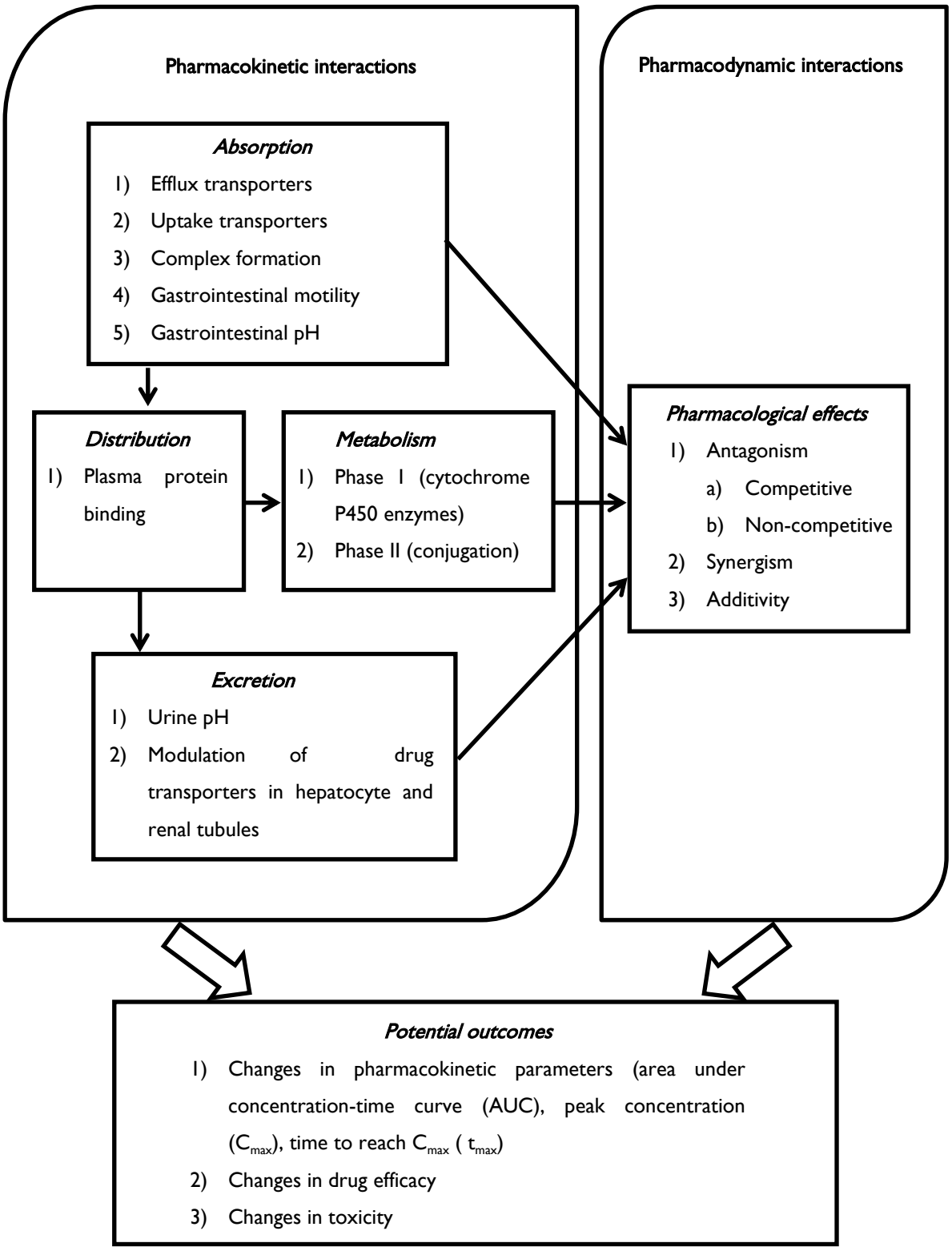


Figure I.1 – Illustrative summary of possible pharmacokinetic and pharmacodynamic herb-drug interaction mechanisms and their common potential outcomes (adapted from TARIRAI, VILJOEN and HAMMAN, 2010).

I.3.1.1 Herb-drug interactions at the level of oral drug absorption

Herbal constituents are known to exert a major effect on intestinal enterocytes due to their high concentrations in the gut lumen (HU *et al.,* 2005; IZZO *et al.,* 2005). The intestinal enterocytes represent the first cell lining limiting the entry of orally administered drugs into the body. Several reports have indicated that human drug efflux transporters and also metabolizing enzymes are expressed at high levels in the villus tip of enterocytes, the primary site of absorption for orally administered drugs. The modulation of intestinal transporter proteins and metabolizing enzymes by herbal medicines represents a potentially important mechanism by which the bioavailability of co-administered drugs can be changed (HU *et al.,* 2005).

Herbal extracts can also change drug concentrations through inhibition or induction of uptake or efflux transporters. The efflux of drugs against a steep concentration gradient is mediated by the adenosine 5'-triphosphate (ATP) binding cassette (ABC) transporters [e.g. P-glycoprotein (P-gp) and multidrug resistance-associated protein-2 (MRP2)] (TARIRAI, VILJOEN and HAMMAN, 2010). P-gp (ABCB1) is a phosphorylated glycoprotein encoded by human *MDR1* gene and murine *mdr1a, mdr1b* and *mdr2* genes. P-gp is an ATP-dependent drug efflux pump that is expressed at high levels on the apical surfaces of epithelial cells in the liver, kidney, pancreas, small intestine, colon, adrenal gland and blood-brain barrier (ZHOU, LIM and CHOWBAY, 2004). P-gp limits the entry of xenobiotics to the systemic circulation from the intestine, promotes its hepatic elimination, minimizes the distribution through several vital organs and limits the reabsorption into the systemic circulation from the kidney tubules (VARMA *et al.,* 2003; OZVEGY-LACZKA *et al.,* 2005). The over-expression of P-gp has been associated with the clinical multi-drug resistance (MDR) phenotype (ZHOU, LIM and CHOWBAY, 2004). It has been suggested that P-gp plays an important role in increasing the exposure of susceptible drugs to cytochrome P450 (CYP) isoenzymes in the intestine through repeated cycling of drug into and out of the gut enterocytes via passive diffusion and active transport by P-gp-mediated efflux (DARWICH *et al.,* 2010). Thus, in physiological conditions P-gp acts as a beneficial functional barrier minimizing the systemic exposure to xenobiotics, but it is also an undesirable barrier against certain pharmacological treatments involving P-gp substrate drugs (MARCHETTI *et al.,* 2007). The activity of P-gp can be inhibited by competitive or noncompetitive mechanisms, which may potentially lead to toxic blood plasma concentrations of drugs that are P-gp substrates. On the other hand, the

induction of efflux transporters may result in sub-therapeutic plasma drug levels leading to treatment failure (TARIRAI, VILJOEN and HAMMAN, 2010).

The organic anion transporting polypeptide 1b1 (OATP1B1) is a genetically polymorphic influx transporter expressed on the sinusoidal membrane of human hepatocytes, and mediates the hepatic uptake of many endogenous compounds as well as of several drugs (NIEMI, PASANEN and NEUVONEN, 2011). Co-administration of aliskiren and grapefruit juice results in reduced bioavailability of aliskiren, which is thought to be due to the inhibition of influx transporters (SEDEN *et al.,* 2010).

The alteration of gastrointestinal motility or the intestinal pH value following consumption of herbal medicines can also have a marked impact on the therapeutic outcomes resulting from the treatment with prescribed drugs. Herbal medicines that induce diarrhea shorten the transit time of the drug along the gastrointestinal tract and reduce the time of contact with the gastrointestinal epithelium, leading therefore to lower drug absorption. For example, *Echinacea purpurea* and *H. perforatum* are two herbs that are known to induce diarrhea and therefore may affect the absorption of drugs. On the other hand, herbal medicines capable to induce constipation may lead to the increase of the residence time of a drug over its absorption window due to prolongation of the transit time of the drug along the gastrointestinal tract (TARIRAI, VILJOEN and HAMMAN, 2010).

The formation of insoluble herb-drug complexes in the gastrointestinal tract can also significantly reduce the bioavailability of drugs and therefore result in sub-therapeutic effects. Fibres such as psyllium and alginates may also chelate iron and drugs such as metformin and glibenclamide as shown in *in vitro* and *in vivo* studies (TARIRAI, VILJOEN and HAMMAN, 2010).

I.3.1.2 Herb-drug interactions at the level of distribution

Given that many herbal components are highly bound by plasma proteins, they may displace the drugs from plasma protein binding sites. However, there is rare clinical evidence for altered protein binding of drugs by herbal medicines. Even so, herbs such as meadow sweet and black willow, which contain pain-reducing salicylates, may displace highly protein-bound drugs such as warfarin and carbamazepine, thus increasing the toxicity of the drugs (KUHN, 2002).

I.3.1.3 Herb-drug interactions at the level of metabolism

The mechanisms responsible for the altered drug concentrations by concomitant administration of herbal medicines are mainly related with the induction or inhibition of hepatic and intestinal drug metabolizing enzymes and/or drug transporters (Figure I.2) (GOUWS *et al.,* 2012).

Herb-drug metabolic interactions can occur in the liver and in extra hepatic tissues such as the lung, kidney, gastrointestinal tract and gut mucosa due to the presence of CYP isoenzymes (TARIRAI, VILJOEN and HAMMAN, 2010). The phase I and phase II enzymes are responsible for the metabolism of constituents of herbal medicines as well as prescribed drugs. Among these enzymes, the CYP isoenzymes are the major catalysts involved in phase I drug biotransformation reactions for xenobiotic metabolism. The CYPs family is a large and diverse group of hemeproteins. The human CYPs are membrane-associated proteins that are located either in the inner membrane of mitochondria or in the endoplasmic reticulum of cells. In humans, CYPs are the major enzymes involved in drug metabolism and bioactivation, and they are responsible for almost 75% of the total drug metabolism (WANG and CHOU, 2010). The CYPs catalyze reactions such as hydroxylation, dealkylation, oxidation, and others, which usually increase the polarity of substrate molecules and facilitate their excretion (IOANNIDES and LEWIS, 2004).

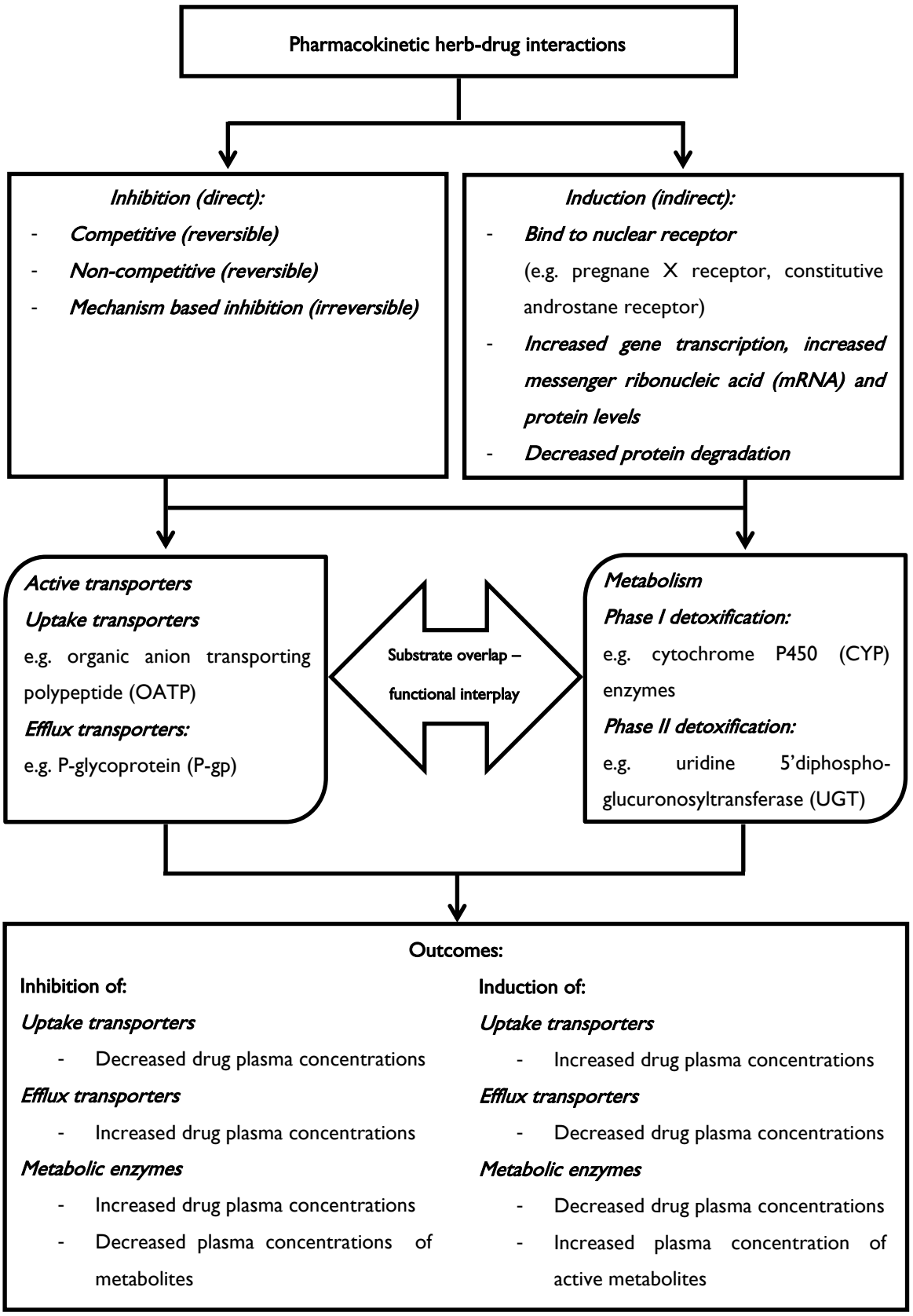


Figure I.2 – Mechanisms and outcomes of pharmacokinetic herb-drug interactions involving drug metabolizing enzymes and transporters (adapted from GOUWS *et al.,* 2012).

Herbal extracts can change drug metabolism through inhibition of hepatic and intestinal CYP isoenzymes, by means of reversible (competitive or noncompetitive) and irreversible inhibitory mechanisms (GOUWS *et al.,* 2012). Consequently, inhibitory effects on a specific drug metabolizing system results in increased plasma concentrations of the substrate compound and, therefore, it can lead to drug toxicity (MARKOWITZ and ZHU, 2012). In general, competitive inhibition is dose dependent and usually occurs within 24 h of ingestion of the inhibitor, although the time to reach maximal inhibition depends on the half-life time of the affected drug and the inhibiting agent. When the inhibitor is discontinued, reestablishment of the baseline conditions is also dependent on the elimination rate of the affected drug and the inhibitor (SPINA, SANTORO and D'ARRIGO, 2008). Grapefruit juice (*Citrus paradisi*) affects the drug concentrations of several drugs including felodipine, nifedipine, simvastatin, lovastatin, losartan, midazolam, triazolam, terfenadine and cyclosporine. These interactions resulted in most of the cases in an increased drug exposure and they were generally mediated through inhibition of intestinal CYP enzymes, namely CYP3A4. The furanocoumarins present in grapefruit juice (e.g. 6',7'-dihydroxybergamottin and bergamottin) have been proposed as active constituents that are able of modulating CYP3A4 (SEDEN *et al.,* 2010).

On the other hand, the induction of CYP isoenzymes can occur through binding to nuclear receptors, increasing the gene transcription, messenger ribonucleic acid (mRNA) and protein levels and decreasing protein degradation (GOUWS *et al.,* 2012). Enzyme induction is a slow regulatory process that is dose and time dependent. The extent of induction is generally proportional to the dose of the inducing agent; because the process usually requires synthesis of the new enzyme, induction occurs with some delay after exposure to the inducing agent, generally from a few days to 1-2 weeks (SPINA, SANTORO and D'ARRIGO, 2008). Modulation of phase II metabolic enzymes, such as uridine 5'diphospho-glucuronyltransferases (UGT), can also result in drug interactions. Enzyme induction can have a profound effect on the pharmacokinetics of drugs metabolized by the susceptible enzyme, as it can lead to decreased plasma concentrations of an active compound and possibly to loss of clinical efficacy. In addition, herb-drug interactions are not limited to effects on either metabolizing enzymes or transporters alone but may involve a concerted effect on both. Therefore, this can result in complex drug interactions that are difficult to predict or manage and may occur when concurrent inhibition/induction of either metabolizing enzymes or transporters is experienced. This interplay due to overlap in

substrate specificity between metabolizing enzymes and efflux transporters has been identified as a complicating factor in the explanation of herb-drug interactions (GOUWS *et al.,* 2012). Several studies showed that *H. perforatum* affects the pharmacokinetics of many drugs by inducing P-gp transporter and CYP isoenzymes, such as CYP3A4, CYP2C19, and CYP2C9. *H. perforatum* decreases the plasma levels of alprazolam, amitriptyline, cyclosporine, digoxin, fexofenadine, imatinib mesylate, indinavir, irinotecan, methadone, oral contraceptives, simvastatin and tacrolimus (VENTOLA, 2010).

I.3.1.4 Herb-drug interaction at the level of excretion

Some herbs can also change the renal function and consequently may change the excretion of some drugs. For example, St. John´s wort may increase the renal excretion of digoxin, which is mediated by P-gp (JOHNE *et al.,* 1999).

The pharmacokinetic herb-drug interactions may be clinically relevant when considerable changes occur in pharmacokinetic parameters of the drug concomitantly administered with the herbal product. Pharmacokinetic parameters such as area under the concentration-time curve (AUC), peak plasma concentration (C_{max}) and the time to reach C_{max} (t_{max}) are directly related to the drug's efficacy and toxicity (TARIRAI, VILJOEN and HAMMAN, 2010) (Figure I.1). In addition, some examples of clinically relevant herb-drug interactions are summarized in Table I.1. From the information contained in Table I.1, it is evident that several cardiovascular drugs of narrow therapeutic range such as digoxin, warfarin and also AM were affected by herbal extracts.

Table I.1 – Some examples of clinically relevant pharmacokinetic herb-drug interactions.

Herb	Drug	Subjects	Study design	Clinical outcome	Interaction mechanism	Reference
Allium sativum	Saquinavir	10 healthy volunteers	Two-treatment, three period, single sequence, longitudinal study	↓ AUC_{0-8}; ↓ through concentrations at 8 h after dosing; ↓ C_{max}	CYP3A4 and/or P-gp induction	PISCITELLI *et al.,* 2002
American ginseng	Warfarin	20 healthy volunteers	Randomized double-blind, placebo-controlled trial	↓ INR AUC; ↓ C_{max}; ↓ AUC	Hepatic enzymes induction	YUAN *et al.,* 2002
Cranberry juice	Midazolam	16 healthy volunteers (8 men and 8 women)	Prospective randomized, crossover, open-label study	↑ $AUC_{0-\infty}$; ↓ 1'-hydroxymidazolam/midazolam $AUC_{0-\infty}$ ratio	Enteric CYP3A inhibition	NGO *et al.,* 2009
Ergoloid mesylates	Ticlopidine	8 healthy volunteers	Sequential 3-phase study	↑ AUC_{0-12}; ↑ C_{max}	OATP-B inhibition	LU, HUANG and LAI, 2006
Ginko biloba	Talinolol	12 healthy volunteers	Open crossover study	↑ AUC_{0-8}; ↑ C_{max}; ↑ $t_{1/2el}$	P-gp inhibition	FAN *et al.,* 2009
Grapefruit juice	Amiodarone	11 healthy volunteers	Single sequence, repeated-measures study	↑ AUC; ↑ C_{max}	CYP3A4 inhibition	LIBERSA *et al.,* 2000
Grapefruit juice	Felodipine	6 men a 2 women	Randomized, 4-way crossover study	↑ AUC; ↑ C_{max}; ↓ dehydrofelodipine/felodipine ratio	CYP3A4 inhibition	BAILEY, DRESSER and BEND, 2003
Green or black tea	Folic acid	7 healthy volunteers	Open-labeled randomized crossover study	With 0.4 mg of folic acid: ↓ C_{max} of serum folate; ↓ $AUC_{0-\infty}$ With 5 mg of folic acid: ↓ C_{max} of serum folate; ↓ $AUC_{0-\infty}$ with green tea	-	ALEMDAROGLU *et al.,* 2008
Green tea catechins	Buspirone	42 healthy volunteers	Crossover study	↑ AUC	CYP3A4 inhibition	CHOW *et al.,* 2006
Hypericum perforatum	Alprazolam and dextromethorphan	12 healthy volunteers	Open label crossover study	↑ AUC; ↑ clearance; ↓ $t_{1/2el}$ alprazolam; ↓ urinary ratio of dextromethorphan	CYP3A4 induction	MARKOWITZ *et al.,* 2003
Hypericum perforatum	Cyclosporine	10 renal transplanted patients	Crossover study	↓ AUC_{0-12}; ↓ peak plasma concentration and at the end of one dosing interval	-	MAI *et al.,* 2004
Hypericum perforatum	Digoxin	18 young adults	-	↓ AUC_{0-3}; ↓ AUC_{0-24}; ↓ C_{max}	P-gp induction	GURLEY *et al.,* 2008

Table I.1 – Some examples of clinically relevant pharmacokinetic herb-drug interactions *(cont.).*

Herb	Drug	Subjects	Study design	Clinical outcome	Interaction mechanism	Reference
Hypericum perforatum	Bupropion	80 health male subjects	Open-label, two phase study	↓ $AUC_{0-\infty}$; ↑ oral clearance of bupropion; ↓ $t_{1/2el}$ hydroxybupropion	CYP3A4 induction	LEI *et al.,* 2010
Hypericum perforatum	Finasteride	20 healthy men	Open-label, fixed order, single center study	↓ C_{max}; ↓ AUC_{0-24}; ↓ $t_{1/2el}$	Metabolism induction	LUNDAHL *et al.,* 2009
Salvia miltiorrhiza	Midazolam	12 healthy volunteers	Sequential open-label, two-period study	↑ apparent oral clearance; ↓ C_{max}; ↓ $AUC_{0-\infty}$	CYP3A4 induction	QIU *et al.,* 2010
Schisandra chinensis	Talinolol	12 healthy volunteers	Open crossover study	↑ AUC_{0-8}; ↑ C_{max}; ↑ $t_{1/2el}$	P-gp inhibition	FAN *et al.,* 2009
Schisandra sphenanthera	Tacrolimus	12 healthy male volunteers	-	↑ AUC; ↑ AUMC; ↑ C_{max}; ↓ CL/F; ↓ V/F	-	XIN *et al.,* 2007
Schisandra sphenanthera	Midazolam	12 healthy volunteers	-	↑ AUC; ↑ AUMC; ↑ C_{max}; ↑ t_{max} midazolam; ↑ t_{max} 1'-hydroxymidazolam	CYP3A inhibition	XIN *et al.,* 2009

AUMC, area under the moment curve; AUC, area under the concentration-time curve; AUC_{0-t}, area under the concentration-time curve from zero to the last sampling time; $AUC_{0-\infty}$, area under the concentration-time curve from zero to infinite; CL/F, clearance normalized by bioavailability; C_{max}, peak concentration; INR, international normalized ratio; OATP-B, organic anion transporting polypeptide B; $t_{1/2}$, apparent terminal elimination half-life time; V/F, volume of distribution normalized by bioavailability.

I.4. AMIODARONE

AM ($C_{25}H_{29}I_2NO_3$) [2-*n*-butyl-3-(3,5-diiodo-4-diethylaminoethoxy-benzoyl)-benzofuran (Figure I.3)], a diiodinated benzofuran derivative was introduced into clinical practice as an antianginal agent in 1962, and in 1967 was developed for the treatment of ventricular and supraventricular tachyarrhythmias. Indeed, AM is one of the most widely used antiarrhythmic agents because of its high effectiveness (PAPIRIS *et al.,* 2010), which is currently approved for the treatment of life-threatening recurrent ventricular arrhythmias, such as ventricular fibrillation or ventricular tachycardia, whenever other treatments are ineffective or have not been tolerated (PAPIRIS *et al.,* 2010; VAN HERENDAEL and DORIAN, 2010); this drug is also the antiarrhythmic agent most commonly used for the treatment of atrial fibrillation due to its superior efficacy over other drugs for maintaining normal sinus rhythm (GOLDSCHLAGER *et al.,* 2007; PAPIRIS *et al.,* 2010; BRENNER and DELACRÉTAZ, 2011; SCHWEIZER *et al.,* 2011).

AM has long been recognised as a drug having a narrow therapeutic window (STÄUBLI, 1983; POLLAK, BOUILLON and SHAFER, 2000). Nevertheless, probably due to its atypical pharmacokinetic properties that are discussed below, and in contrast to other narrow therapeutic index drugs, the data on the clinical value of the use of AM plasma concentrations for routine therapeutic drug monitoring (TDM) have been conflicting (VAN HERENDAEL and DORIAN, 2010). Some studies have suggested no correlation between plasma/serum concentration levels of AM and its efficacy or toxicity (ESCOUBET *et al.,* 1986; CAMPBELL and WILLIAMS, 2001). On the other hand, some authors have shown that serious AM-induced toxicity is more likely at serum/plasma drug concentrations above 2.5 µg/mL (ROTMENSCH *et al.,* 1984; POLLAK, and SHAFER, 2004). ROTMENSCH *et al.* (1983) also concluded that serum concentrations of AM below 2.5 µg/mL significantly improve the AM's benefit-to-risk relationship. In spite of the lack of scientific consensus on this issue, different therapeutic plasma/serum concentration ranges for AM have been reported in literature, but the more typically accepted concentration range is 0.5-2.0 µg/mL (LESNE and PELLEGRIN, 1987; HUY *et al.,* 1991; MANFREDI *et al.,* 1995; IERVASI *et al.,* 1997; PÉREZ-RUIZ *et al.,* 2002; SHAYEGANPOUR, HAMDY and BROCKS, 2008). Indeed, in the absence of a loading-dose period, the monitoring of AM plasma concentrations at the beginning of treatment is expected to be unhelpful or misleading since a long time (4- to 6- week) is required to reach the steady-state concentrations (SIDDOWAY, 1983); in fact, AM is a highly lipophilic drug presenting,

therefore, a marked deposition in tissues and, consequently, a large apparent volume of distribution and a long elimination half-life (HOSAKA, 2002). However, the monitoring of AM plasma concentrations may be of great value for the follow-up of patients with arrhythmias chronically treated with AM, differentiating treatment failure from suboptimal dosing and reducing the incidence of concentration-related adverse effects (ROTMENSCH *et al.,* 1984). Thus, the availability of rapid, sensitive and reliable analytical methods for the determination of AM and its metabolites in biological matrices (particularly, plasma or serum) could be considered crucial to support the routine TDM in clinical practice. Furthermore, bioanalytical methods appropriately developed and fully validated to quantify AM and its metabolites in biological samples obtained from humans and different animal species also play an important role to support clinical and non-clinical pharmacokinetic studies, as well as to investigate, in more detail, the metabolic pathways of AM. Actually, despite the clinical use of AM for more than 40 years, new insights have been recently gained in the understanding of the metabolism and toxicity of the drug (HA *et al.,* 2001a*,* 2001b*,* 2005; DENG *et al.,* 2011; ZAHNO *et al.,* 2011).

I.4.1 PHARMACOKINETIC PROPERTIES

In humans AM has unusual and complicated pharmacokinetic properties likely because of its high lipid solubility. After oral administration, AM presents a variable bioavailability (20-80%) and the C_{max} is reached within 3-7 h following oral dosing (VAN HERENDAEL and DORIAN, 2010). In addition, AM undergoes extensive enterohepatic recirculation before entry into the central compartment (systemic blood circulation) (FREEDMAN and SOMBERG, 1991) and it is also widely bounded (approximately 99.9%) to plasma proteins (VERONESE, MCLEAN and HENDRIKS, 1988). Following the systemic absorption, AM is extensively distributed to the body tissues with exceptionally high tissue/plasma partition coefficients (FREEDMAN and SOMBERG, 1991; OHYAMA *et al.,* 2000; VAN HERENDAEL and DORIAN, 2010). A three-compartment model seems to be the best to explain the AM's kinetics (VAN HERENDAEL and DORIAN, 2010). The distribution half-life ($t_{1/2alpha}$) of AM out of the central compartment to peripheral and deep tissue compartments may be as short as 4 h (FREEDMAN and SOMBERG, 1991). After the initial distribution phase (alpha phase), the highest levels of AM have been found in adipose tissue, lung, liver, and lymph nodes, which represent the deep compartment; on the other hand, the lowest concentrations of

AM have been observed in brain, muscles and thyroid, being part of the called peripheral compartment (VAN HERENDAEL and DORIAN, 2010). Consequently, due to the variable and extensive uptake into different tissues, AM is characterised by a very high apparent volume of distribution (V_d) (5000 L) and an extremely long elimination half-life (SHAYEGANPOUR, HAMDY and BROCKS, 2008; PAPIRIS *et al.,* 2010). In particular, the terminal elimination half-life ($t_{1/2beta}$) has shown to be long and variable (9-77 days) secondary to the slow elimination of the drug from the body tissues, primarily from fat tissue (FREEDMAN and SOMBERG, 1991).

From the metabolic point of view, it has been accepted that AM undergoes extensive first-pass hepatic metabolism in humans (VAN HERENDAEL and DORIAN, 2010). Mono-*N*-desethylamiodarone (MDEA) is the major metabolite of AM and it was the only known metabolite until the beginning of the 21st century (HA *et al.,* 2001a). MDEA is a product of the most predominant metabolic route, *N*-deethylation, catalyzed by CYP isoenzymes. This metabolite is pharmacologically and toxicologically active and may contribute to the overall antiarrhythmic and toxic effects ascribed to the parent compound (SOYAMA *et al.,* 2002; TRIVIER *et al.,* 1993). However, AM may also be metabolised through other minor metabolic pathways, such as *O*-dealkylation, hydroxylation, deiodination, deamination, ω-carboxylation and glucuronidation (SOYAMA *et al.,* 2002; HA *et al.,* 2005; SHAYEGANPOUR, EL-KADI and BROCKS (2006); LOHMANN *et al.,* 2008; DENG *et al.,* 2011). HA *et al.* (2005) suggested that MDEA may be further cleared by hydroxylation to *n*-3-hydroxybutyl mono-*N*-desethylamiodarone (3'OH-MDEA), dealkylation to di-*N*-desethylamiodarone (DDEA) and deamination to deaminated AM.

More recently, using a modern bioanalytical methodology (ultraperformance liquid chromatography/quadrupole time-of-flight mass spectrometry), DENG *et al.* (2011) identified several novel AM metabolites in human bile; overall, 33 metabolites of AM were revealed in human bile, including 22 phase I and 11 phase II metabolites. In Figure I.3 are summarized the metabolic pathways for AM.

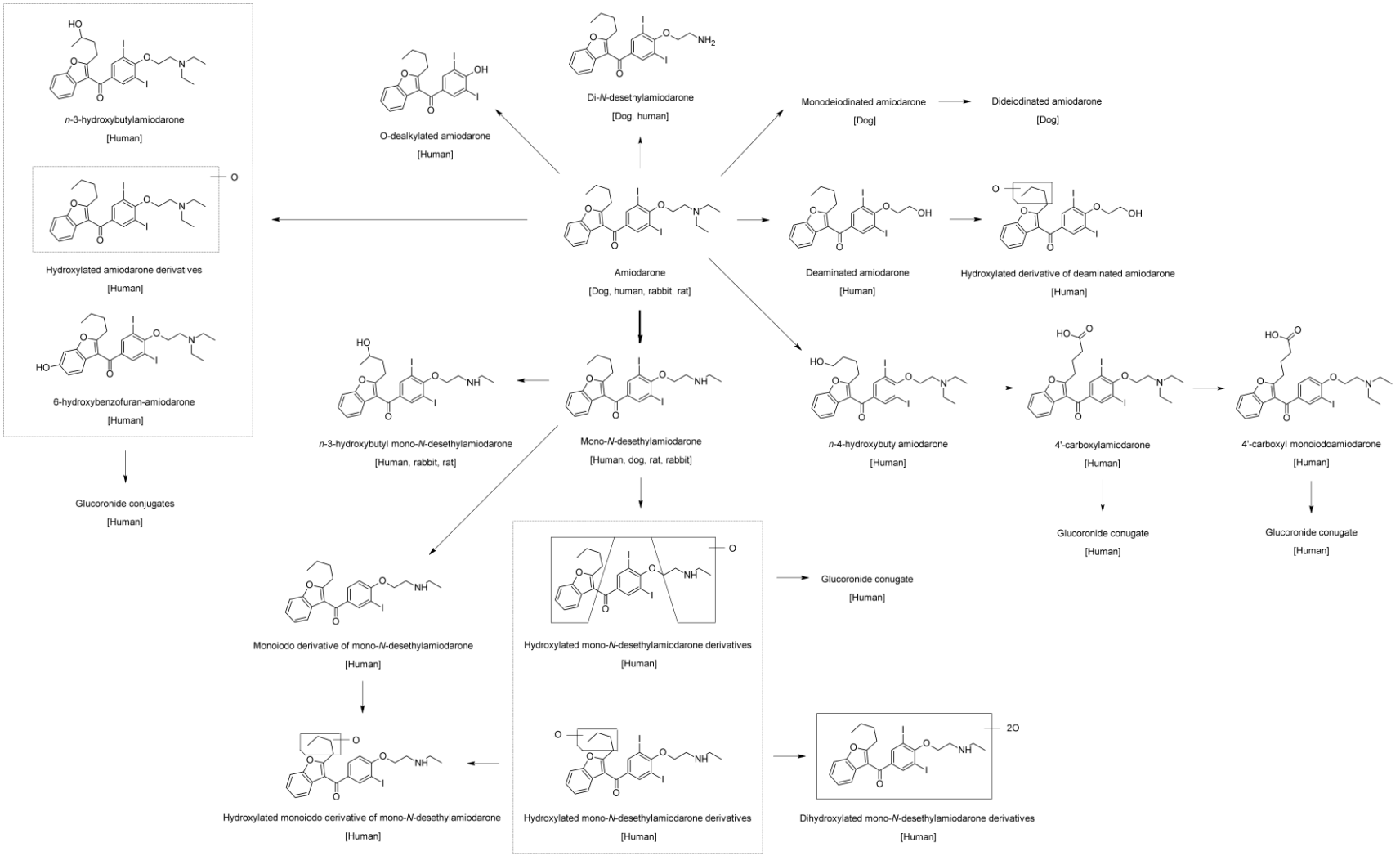


Figure I.3 – Metabolic pathways for amiodarone in human and animal species (dog, rabbit and rat) (HA *et al.,* 2001a, 2001b, 2005; DENG *et al.,* 2011).

In fact, DENG *et al.* (2011) identified as major metabolites in human bile the MDEA and a novel metabolite 4'-carboxylamiodarone. This novel metabolite was biotransformed by oxidation of the *n*-butyl chain of AM by CYP isoenzymes to the ω-hydroxyl intermediate, followed by oxidation by alcohol dehydrogenase. The large variability of AM pharmacokinetics in humans may be explained by the greater insight of CYP isoenzymes and transporters involved in the formation and elimination of the metabolite 4'-carboxylamiodarone. Despite some doubts about the existence of a deiodination pathway in previous studies, this minor pathway was confirmed by the presence of deiodinated metabolites in human bile. Furthermore, the glucuronidation of AM and of its phase I metabolites was identified as the most important conjugation pathway. The glucuronidation of AM together with biliary excretion was thought to be the final elimination process for AM (DENG *et al.,* 2011).

Despite pharmacokinetic parameters in rats are comparable with humans some differences exists, the $t_{1/2beta}$ observed in rat was found to be much lower than that found in humans, reflecting the lower V_d and higher clearance (CL) in rats (SHAYEGANPOUR, HAMDY and BROCKS, 2008). In addition, lower concentration levels of MDEA were reported in rats compared to humans (WYSS, MOOR and BICKEL, 1990; SHAYEGANPOUR, HAMDY and BROCKS, 2008) that may be related with the higher CL values of MDEA than those of the parent drug in the rat. Indeed, there is also a difference in the hepatic extraction ratio of AM, which is higher in the rat than in human (SHAYEGANPOUR, HAMDY and BROCKS, 2008).

In humans and rats has been demonstrated that AM is extensively biotransformed to MDEA by *N*-deethylation (ELSHERBINY and BROCKS, 2010; ELSHERBINY, EL-KADI and BROCKS, 2010; WAN *et al.,* 2011). This major metabolite was further biotransformed to 3'OH-MDEA that was found in plasma, liver, heart, lung and kidney in rats (Figure I.3) (HA *et al.,* 2001a). Despite some differences, the rat shares with humans many of the pharmacokinetic properties of AM and has been regarded as one of the most used models among the small laboratory animals for studying some pharmacological and toxicological aspects concerning the AM (PLOMP *et al.,* 1987, NAJJAR, 2001; ELSHERBINY and BROCKS, 2010).

In the rabbit, some previous reports suggest that other metabolites of AM besides MDEA exist (KOZLIK *et al.,* 2001). However, despite some doubts about the identity of the metabolites formed, it was confirmed that in rabbit liver microsomes MDEA was biotransformed to 3'OH-MDEA (HA *et al.,* 2001b). Indeed, this metabolite (3'OH-MDEA)

was considered to be the major metabolite of MDEA in rabbit liver microsomes (Figure I.3) (HA *et al.,* 2001a; SHAYEGANPOUR, HAMDY and BROCKS, 2008).

In the dog, AM was biotransformed to the major metabolite MDEA being further dealkylated to DDEA (Figure I.3). DDEA was identified in plasma and myocardial tissue of dogs. It was found that this metabolite accumulates in myocardium at a higher extent than the parent drug (AM) (LATINI *et al.,* 1984).

I.4.2 PHARMACODYNAMICS

AM has electrophysiological characteristics of all four Singh Vaughan Williams classes (HAN, WILLIAMS and VANDERPUMP, 2009). The most prominent effect of long-term AM therapy is class III antiarrhythmic activity which prolongs cardiac action potential duration via potassium channel blockade (COHEN-LEHMAN *et al.,* 2010; PAPIRIS *et al.,* 2010). AM decreases conduction velocity by blocking sodium channels (class I activity) (PAPIRIS *et al.,* 2010). AM has also anti-sympathetic properties (class II activity) in which there are non-competitive inhibition of α- and β-adrenergic receptors. Class IV activity produces negative chronotropic effects in nodal tissues via L-type calcium channel blockade. The anti-sympathetic action (Class II activity) and blockade of potassium and calcium channels (Class III and IV) are responsible for the slowing of conduction and prolongation of refractoriness in the sinus and atrioventricular nodes useful in the management of supraventricular arrhythmia (HAN, WILLIAMS and VANDERPUMP, 2009; CONNOLLY, 2013). The class III activity results in increases in prolongation on the rate-corrected QT (QTc) interval and also in atrial and ventricular refractoriness (CONNOLLY, 2013). AM suppresses sympathetic activities (SHIGA *et al.,* 2011) and reduces heart rate during long-term administration almost to the same extent as most β-blockers (SINGH, 1996). MDEA has also similar electrophysiological actions although slightly less potent than those of AM (HAN, WILLIAMS and VANDERPUMP, 2009).

I.4.2.1 Amiodarone-induced toxicity

The wider therapeutic use of AM is limited due to its potential to induce some serious toxic effects, especially pulmonary toxicity, hepatotoxicity and thyroid dysfunction,

which have been reported mainly after long-term therapy (SEKI *et al.,* 2008, LAFUENTE-LAFUENTE *et al.,* 2009; WOLKOVE and BALTZAN, 2009).

The most serious toxic effects associated with AM therapy are related with pulmonary toxicity (SIDDOWAY, 2003). AM provokes both direct and indirect damage of the pulmonary parenchyma (SANTANGELI *et al.,* 2012). The direct toxicity on the lung cells results from direct drug-induced phospholipidosis and the indirect toxicity is related to an immune-mediated hypersensitivity (SIDDOWAY, 2003; SANTANGELI *et al.,* 2012). The pulmonary toxicity induced by AM is more common in men, especially those with preexisting pulmonary diseases (SANTANGELI *et al.,* 2012).

The liver is an ideal environment for AM accumulation due to its high content of fat. Furthermore, the liver has an active role in AM metabolism particularly through the CYP-metabolizing enzymes (SANTANGELI *et al.,* 2012). An elevation of liver transaminase levels (hepatotoxicity biomarkers) is common in patients receiving long-term AM therapy (SIDDOWAY, 2003). Usually, such abnormalities in liver function tests are promptly reversible after AM discontinuation, although some cases of progressive hepatitis have been described even after withdrawal of the drug therapy. Diagnosis of hepatic toxicity is easily established through monitoring of hepatic enzymes during AM treatment, and the current guidelines recommend monitoring every 6 months. A prompt discontinuation of AM is the only available option to solve this pathological condition (SANTANGELI *et al.,* 2012).

In addition, AM is an iodinated benzofuran derivative with a molecular structure that closely resembles that of thyroid hormones. It contains about 37% of organic iodine by weight, from which 10% is deiodinated to yield free iodide. Given the daily maintenance dose of AM between 100 and 600 mg, about 3.5-21 mg of iodide are released into the systemic circulation, equivalent to 35-140-fold excess of the iodine reference daily intake of 100-150 µg (HAN, WILLIAMS and VANDERPUMP, 2009). Hence, the disruption of the thyroid hormone synthesis and auto-regulation owing to excess of iodine concentrations from AM use may occur in some patients leading to either hypothyroidism or hyperthyroidism (thyrotoxicosis) (COHEN-LEHMAN *et al.,* 2010). AM-induced hypothyroidism occurs when the intrathyroidal iodine levels are excessive reaching a critical point. This effect leads to the temporary inhibition of the iodine transport and of the thyroid hormone synthesis until the intrathyroidal stores return to normal levels (HAN, WILLIAMS and VANDERPUMP, 2009). This phenomenon is denominated Wolff-Chaikoff effect (HAN, WILLIAMS and VANDERPUMP, 2009; COHEN-LEHMAN *et al.,* 2010). On the other hand, AM-induced thyrotoxicosis arises due to a

failure of thyroid auto-regulatory mechanism that is thought to lead to thyroid autonomy (COHEN-LEHMAN *et al.,* 2010). This effect occurs by the addition of iodine substrate from AM that leads to excessive thyroid hormone synthesis and thyrotoxicosis (Jod-Basedow effect) (HAN, WILLIAMS and VANDERPUMP, 2009). This effect occurs typically in areas of iodine deficiency and in patients with underlying nodular or autoimmune thyroid disorders (COHEN-LEHMAN *et al.,* 2010).

I.4.3 DRUG-DRUG AND HERB-DRUG INTERACTIONS INVOLVING AMIODARONE

AM has been associated to important clinical drug-drug interactions either of pharmacodynamic and/or pharmacokinetic nature (EDWIN, JENNINGS and KALUS, 2010; KARIMI *et al.,* 2010; ROUGHEAD *et al.,* 2010). Nonetheless, AM has usually been considered the perpetrator or interacting agent in the majority of pharmacokinetic-based drug interactions reported in literature (Table I.2), inducing a change (generally an increase) in serum/plasma levels of co-administered drugs [e.g. digoxin (LESKO, 1989; TRUJILLO and NOLAN, 2000), warfarin (LESKO, 1989; TRUJILLO and NOLAN, 2000; YAMREUDEEWONG *et al.,* 2003), phenytoin (LESKO, 1989), simvastatin (BECQUEMONT *et al.,* 2007; MAROT *et al.,* 2011), cyclosporine (CHITWOOD, ABDUL-HAGG and HEIM-DUTOV, 1993; YAMREUDEEWONG *et al.,* 2003), flecainide (TRUJILLO and NOLAN, 2000) and lidocaine (SIEGMUND, WILSON and IMHOFF, 1993; HA *et al.,* 1996)].

Table I.2 – Major drug-drug interactions involving amiodarone as perpetrator drug.

Drugs	Type of interaction	Mechanism	Effect	Species	Type of study	References
β-adrenoceptor antagonists	Pharmacodynamic	Additive β-adrenoceptor blocking effect	Bradycardia	Human	*In vivo*	BOUTITIE *et al.,* 1999; YAMREUDEEWONG *et al.,* 2003
Calcium channel blockers (verapamil and diltiazem)	Pharmacodynamic	Additive calcium channel blocking effect; negative ionotropic effects	Bradycardia and auriculoventricular blockade	Human	*In vivo*	TRUJILLO and NOLAN, 2000; TAYLOR, 2002; YAMREUDEEWONG *et al.,* 2003; RAMOS *et al.,* 2004
Cyclosporine	Pharmacokinetic	Metabolism inhibition	↑ plasmatic levels	Human	*In vivo*	CHITWOOD, ABDUL-HAGG and HEIM-DUTHOY, 1993; YAMREUDEEWONG *et al.,* 2003
Cisapride	Pharmacodynamic	-	↑ effects on QT interval	Human	*In vivo*	YAMREUDEEWONG *et al.,* 2003
Digoxin	Pharmacokinetic	↓ clearance renal and non-renal; P-gp inhibition in the gastrointestinal tract	↑ serum concentrations	Human	*In vivo*	MARAGNO *et al.,* 1984; NADEMANEE *et al.,* 1984; YAMREUDEEWONG *et al.,* 2003
Flecainide	Pharmacokinetic	Metabolism inhibition	↑ plasma concentrations	Human	*In vivo*	TRUJILLO and NOLAN, 2000; YAMREUDEEWONG *et al.,* 2003
Lidocaine	Pharmacokinetic	Metabolism inhibition	↑ plasma concentrations	Human	*In vivo*	TRUJILLO and NOLAN, 2000; YAMREUDEEWONG *et al.,* 2003
Quinidine	Pharmacokinetic	Effects on the pharmacokinetics	↑ plasma concentrations; ↑ effects on QT interval	Human	*In vivo*	TARTINI *et al.,* 1982
Phenytoin	Pharmacokinetic	Hepatic metabolism inhibition through CYP2C9	↑ serum concentrations	Human	*In vivo*	LESKO, 1989; NOLAN *et al.,* 1989; TRUJILLO and NOLAN, 2000;
Simvastatin	Pharmacokinetic	CYP3A4 inhibition	↑ plasma concentrations; Rhabdomyolysis	Human	*In vivo*	BECQUEMONT *et al.,* 2007; MAROT *et al.,* 2011
Warfarin	Pharmacokinetic	CYP1A2, CYP2C9 and CYP3A4 inhibition	↑ INR	Human	*In vivo*	ALMOG *et al.,* 1985; TRUJILLO AND NOLAN, 2000; YAMREUDEEWONG *et al.,* 2003

CYP, cytochrome P450; INR, international normalized ratio: P-gp, P-glycoprotein.

Effectively, only few studies have been published describing the interference of other compounds on the pharmacokinetics of AM (Table I.3). Focusing on some relevant drug-drug and herb-drug interactions, the systemic exposure to AM and MDEA was significantly reduced by the simultaneous administration of orlistat (ZHI *et al.,* 2003). In addition, grapefruit juice completely inhibited the formation of the major metabolite of AM and increased the AUC and C_{max} by 50% and 84%, respectively, in comparison with the control group. The inhibition of MDEA formation led to a decrease in the alterations caused by AM on PR and QTc intervals (LIBERSA *et al.,* 2000). Accordingly, considering the existence of several drug-drug interactions and this relevant herb-drug interaction between grapefruit juice and AM, it is absolutely pertinent to investigate the effects of other extracts on the pharmacokinetic of AM (object drug). Particularly, considering the scarcity of scientific information available about the safety of herbal slimming medicines/supplements, it is absolutely pertinent to assess the potential for pharmacokinetic interactions between relevant herbal weight loss extracts and drugs required in multiple cardiovascular diseases. Therefore, it is justified to characterize the interference of standardized herbal extracts commonly used in weight loss programs *(e.g. Fucus vesiculosus, Paullinia cupana, Citrus aurantium* and *Carica papaya)* on the pharmacokinetic profile of AM.

Table I.3 – Major drug-drug and herb-drug interactions involving amiodarone as object drug.

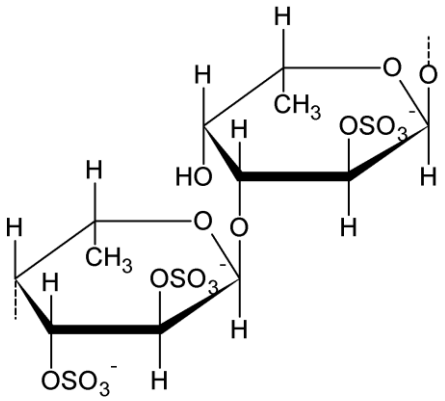
Drugs/Herbs	Type of interaction	Mechanism	Effect	Species	Type of study	References
β-naphthoflavone	Pharmacokinetic	CYP1A1 induction	↓ concentrations of AM in plasma, lung, liver, kidney, heart and intestine; ↑ concentrations of MDEA in plasma, lung, liver, kidney and heart	Rat	*In vivo*	ELSHERBINY and BROCKS, 2010
Cimetidine	Pharmacokinetic	CYP isoenzymes inhibition	↑ plasmatic levels of AM	Human	*In vivo*	KOWEY *et al.,* 1999; TAYLOR, 2002
Cholestyramine	Pharmacokinetic	Binding to AM and ↓ its availability for gastrointestinal absorption	↓ gastrointestinal absorption of AM; ↓ plasmatic levels of AM; ↓ enterohepatic elimination of AM	Human	*In vivo*	NITSCH and LÜBERITZ, 1986; YAMREUDEEWONG *et al.,* 2003
Phenytoin	Pharmacokinetic	Metabolism induction of AM	↓ serum concentrations of AM; ↑ serum concentrations of MDEA	Human	*In vivo*	NOLAN *et al.,* 1990
Indinavir	Pharmacokinetic	CYP3A4 inhibition	↑ serum concentrations of AM	Human	*In vivo*	YAMREUDEEWONG *et al.,* 2003
Orlistat	Pharmacokinetic	↓ gastrointestinal absorption; ↓ fat absorption	↓ plasma concentrations of AM and MDEA	Human	*In vivo*	ZHI *et al.,* 2003
Rifampicin	Pharmacokinetic	CYP3A4 induction	↓ plasmatic levels of AM and MDEA	Human	*In vivo*	ZAREMSBSKI *et al.,* 1999; TAYLOR, 2002
Grapefruit juice	Pharmacokinetic	Metabolism inhibition of AM	↑ plasmatic concentrations of AM; ↓ plasmatic levels of MDEA	Human	*In vivo*	LIBERSA *et al.,* 2000

AM, amiodarone; CYP, cytochrome P450; MDEA, mono-*N*-desethylamiodarone.

I.5. *FUCUS VESICULOSUS*

F. vesiculosus L. (Phaeophyta), also known as bladderwrack or kelp, is a brown-colored, perennial, dioecious edible seaweed that forms dense belts in cold rocky littoral habitats covering large areas from a few decimeters below the water surface to a depth of several meters (DÍAZ-RUBIO, PÉREZ-JIMÉNEZ and SAURA-CALIXTO, 2009). *F. vesiculosus* is a member of the family Fucaceae belonging to the order Fucales (PARYS *et al.,* 2010). This brown seaweed is commonly found on the littoral coast of Northern Atlantic Ocean, the Pacific Ocean and the Baltic Sea (FUJIMURA *et al.,* 2002). The distribution of *Fucus* species in Portuguese Northwestern coastal zone seems to be first determined by the presence or absence of rocky substrata (CAIRRÃO *et al.,* 2004). Several factors affect its growth such as substrate firmness exposure to ice and waves, salinity, wave force, light or competition among macroalgae (DÍAZ-RUBIO, PÉREZ-JIMÉNEZ and SAURA-CALIXTO, 2009).

The major components that give the *Fucus* its reported health benefits are non-digestible polysaccharides (dietary fibre) and polyphenols (DÍAZ-RUBIO, PÉREZ-JIMÉNEZ and SAURA-CALIXTO, 2009). Dietary fibre of *F. vesiculosus* is composed of fucans, alginates, laminaranes and cellulose, with fucoidan (Figure I.4) to be the predominant polysaccharide (RIOUX, TURGEON and BEAULIEU, 2007). The raw *Fucus* powder is rich in both soluble (10.52%) and insoluble (48.63%) fractions of dietary fibre (DÍAZ-RUBIO, PÉREZ-JIMÉNEZ and SAURA-CALIXTO, 2009).



Fucoidan

Figure I.4 – Chemical structure of fucoidan from *Fucus vesiculosus* (PIELESZ and KULEC, 2010).

This brown alga also contains three types of phlorotannins that are termed fucols, where phloroglucinol units are connected by aryl-aryl bonds, fucophlorethols with ether and

aryl-aryl bonds, and phlorethols containing only ether bonds. From *F. vesiculosus* fifteen fucophlorethols with three to eight units of phloroglucinol and four fucols with two to four units of phloroglucinol were described (PARYS *et al.,* 2010). Some phlorotannin oligomers, including trifucodiphlorethol A, trifucotriphlorethol A, fucotriphlorethol A, tetrafucol A and fucophlorethol A (PARYS *et al.,* 2010; LIU and GU, 2012) have been isolated from *F. vesiculosus* (Figure I.5). The concentration in phlorotannins is genetically variable (KOIVIKKO *et al.,* 2008) and is directly dependent on nutrient concentration in the environment. The phlorotannins are a toxic group of substances that are produced as a mean of protection against predators because *Fucus* species are a source of food for many herbivores (CAIRRÃO *et al.,* 2004). Some of the polyphenols of *F. vesiculosus,* namely the phlorotannin oligomers trifucodiphlorethol A and trifucotriphlorethol A (Figure I.5), were recently found to inhibit drug-metabolizing enzymes like CYP1A (PARYS *et al.,* 2010).

F. vesiculosus has also in its composition proteins, minerals, iodine, vitamins and monounsaturated and polyunsaturated fatty acids (DÍAZ-RUBIO, PÉREZ-JIMÉNEZ and SAURA-CALIXTO, 2009).

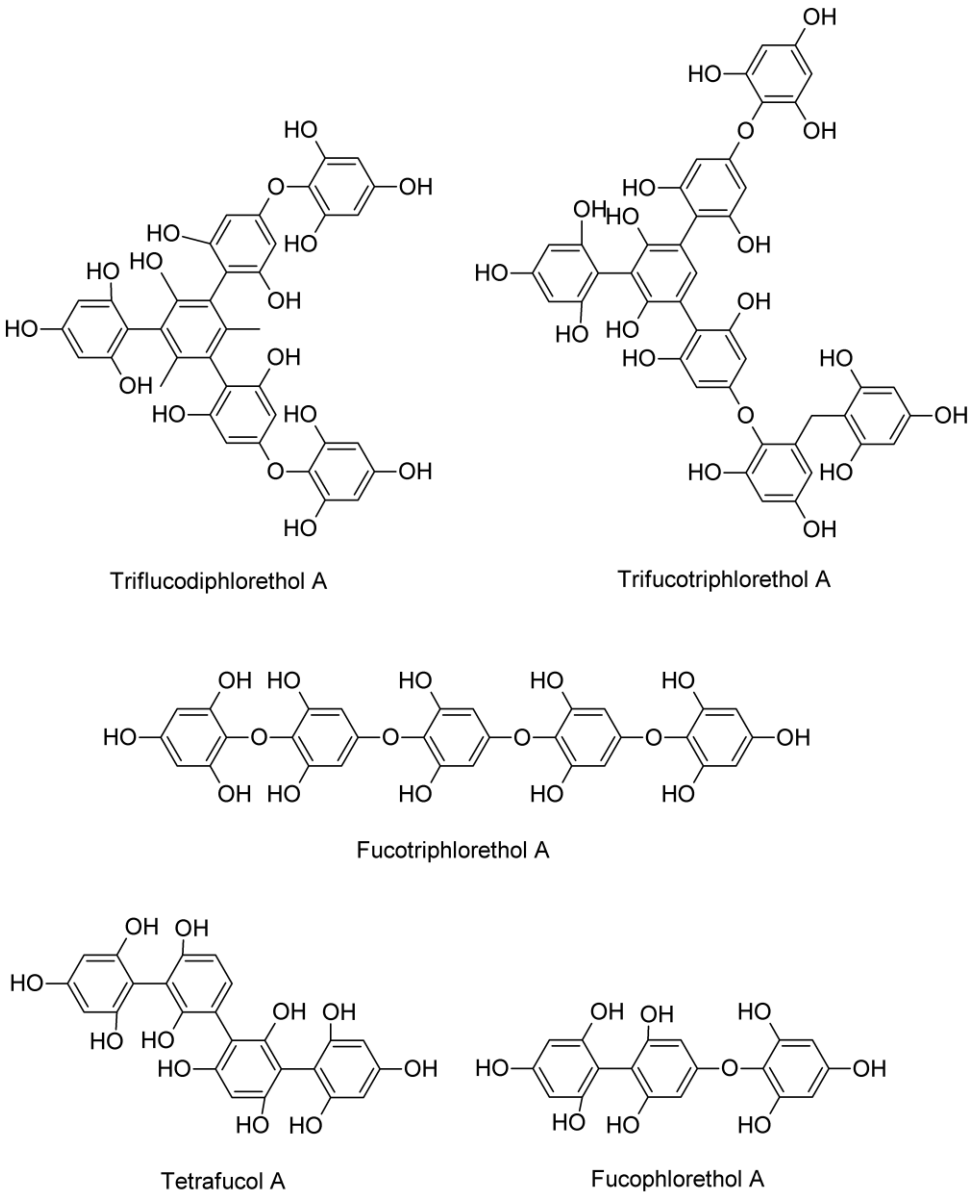


Figure I.5 – Chemical structures of phlorotannin oligomers isolated from *Fucus vesiculosus*: trifucodiphlorethol A, trifucotriphlorethol A, fucotriphlorethol A, tetrafucol A and fucotriphlorethol A (PARYS *et al.,* 2010; LIU and GU, 2012).

F. vesiculosus is claimed to be useful for the treatment of obesity, mainly due to its high levels in iodine, whose action is thought to be related to the stimulation of the thyroid gland and the subsequent effect on metabolic rate. Although iodine is considered the most prominent active ingredient of *F. vesiculosus*, the mucilage (dietary fibre), phytosterols and tetraterpenes are also important constituents responsible for its use in obesity management (MORO and BASILE, 2000; DÍAZ-RUBIO, PÉREZ-JIMÉNEZ and SAURA-CALIXTO, 2009). It is well known that aqueous extracts of this alga have shown various biological properties such as a potent anticoagulant activity (FUJIMURA *et al.,* 2002). The presence of antioxidants and

functional components of dietary fibre in *F. vesiculosus* has promoted the development of a large number of functional ingredients and dietary supplements derived from this seaweed such as fucoidan powders, *F. vesiculosus* capsules or *F. vesiculosus* antioxidant extracts (DÍAZ-RUBIO, PÉREZ-JIMÉNEZ and SAURA-CALIXTO, 2009).

Several properties of the *F. vesiculosus* with application in several areas have been discovered. In particular, strong adsorption effects were observed on the mucus membranes due to the presence of polysaccharides from *F. vesiculosus*. These results suggest that these adsorption effects may account for the therapeutic effects of mucilage-containing plants in the treatment of irritated buccal membranes (SCHMIDGALL, SCHNETZ and HENSEL, 2000). *F. vesiculosus* presents a complex carbohydrate specific lectin-like mucopolysaccharide. This complex carbohydrate was found to agglutinate *Candida guilliermondii* cells. A toxic effect of a lectin-like algal mucopolysaccharide from *F. vesiculosus* has been found to be exerted only on *Escherichia coli* and *Neisseria meningitidis* strains (OLIVEIRA *et al.,* 2003).

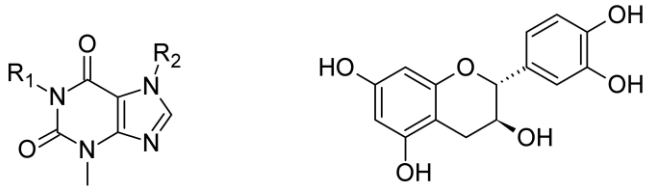
The fucoidan, a sulfated-polysaccharide present in *F. vesiculosus s*howed to promote the contraction of collagen gels (FUJIMURA *et al.,* 2000a). The mechanism of the promotion of gel contraction was related to an increased expression of integrin molecules on the fibroblasts surface (FUJIMURA *et al.,* 2000b). In a study conducted by FUJIMURA *et al.* (2002) was investigated the topical application of an aqueous extract of *F. vesiculosus* on the thickness and the mechanical properties of human skin. It was observed a significant improvement in the elasticity, suggesting that this extract possesses anti-aging activities and should be useful for a variety of cosmetics (FUJIMURA *et al.,* 2002).

Several *Fucus* species have also demonstrated to be good biomarkers of environmental contamination through the enzymes of glutathione-*S*-transferase (GST). CAIRRÃO *et al.* (2004) investigated the activity of *Fucus* species as biomarkers using the family of GST enzymes, because they participate in a mechanism of detoxification catalyzing the conjugation of several xenobiotics with glutathione. As the presence of higher GST levels indicates a more contaminated site, it is possible to discriminate areas with different levels of pollution (CAIRRÃO *et al.,* 2004).

In addition, *F. vesiculosus* significantly increased the total number of days of the menstrual cycle, reduced circulating 17-β-estradiol levels, and elevated serum progesterone levels in premenopausal women with abnormal menstrual cycling histories. Furthermore, *F. vesiculosus,* as well as other brown kelp species, also induced a reduction of the plasma cholesterol levels (SKIBOLA, 2004).

I.6. *PAULLINIA CUPANA*

P. cupana, also known as guarana, is a climbing plant of the family of Sapindaceae, native to the Amazon basin and cultivated in Brazil and Venezuela for its fruits (CAMPOS *et al.,* 2011; PORTELLA *et al.,* 2013). The seeds are rich in methylxanthines (caffeine, theophylline and theobromine) and also contain catechins (Figure I.6), epicatechins, saponins, tannins and proanthocyanidols, as well as trace concentrations of other compounds (KREWER *et al.,* 2011; KLEIN, LONGHINI and DE MELLO, 2012).



R_1 CH_3, R_2 CH_3 Caffeine
R_1 CH_3, R_2 H Theophylline
R_1 H, R_2 CH_3 Theobromine

(+)-Chatechin

Figure I.6 – Chemical structures of the major active compounds of *Paullinia cupana*: caffeine, theophylline, theobromine and (+)-catechin (HEARD, JOHNSON and MOSS, 2006).

Several epidemiological and experimental investigations have described an association between the consumption of other foods rich in catechins and caffeine (coffee and tea) and a lower risk of morbidity and mortality from stroke, coronary heart disease and other causes. A possible causal factor associated with tea and coffee consumption is the role of bioactive compounds present in these foods in metabolic pathways related to body weight loss and a consequent reduction of the overall risk for developing metabolic syndrome (PORTELLA *et al.,* 2013).

Guarana consumption is increasing in the world because it takes part in the composition of many commercial dietary supplements as a weight loss promoting adjuvant (LIMA *et al.,* 2005). Guarana extract is used as stimulant of the central nervous system in cases of physical and mental stress, and as antidiarrheal, diuretic and antineuralgic (KLEIN, LONGHINI and DE MELLO, 2012), as well as in the therapeutics of depression, fatigue and migraine (LIMA *et al.,* 2005). Its high caffeine content and stimulating properties makes *P. cupana* particularly attractive in weight loss programs, since it helps to increase the metabolic rate and can enhance thermogenesis. Indeed, *P. cupana* containing products are popular among athletes because of their ergogenic and "fat burning" effects (LIMA *et al.,*

2005; BULKU *et al.,* 2012). Previous experimental investigations have suggested that guarana has positive effects on lipid metabolism (LIMA *et al.,* 2005), in body weight loss (BOOZER *et al.,* 2001) and increases basal energy expenditure (BÉRUBÉ-PARENT *et al.,* 2005). Furthermore, studies suggested that guarana exhibits a cardioprotective effect by inhibiting platelet aggregation (BYDLOWSKI, YUNKER and SUBBIAH, 1988; PORTELLA *et al.,* 2013). All these positive effects may contribute to reduce the risk factors for cardiovascular diseases (PORTELLA *et al.,* 2013). However, as the consumption of guarana is growing in many countries, more studies on its functional properties are needed.

Even so, a controlled study was recently performed to analyse the association between the habitual consumption of guarana and the prevalence of cardiometabolic diseases (obesity, hypertension, type 2 diabetes and dyslipidemia) in an elderly Amazonian population. The study suggests a lower prevalence of hypertension, obesity and metabolic syndrome in the subjects which self-reported the regular guarana consumption than in those subjects who never reported the ingestion of guarana. Additionally an association was found between guarana consumption and lower cholesterol (total and LDL) and advanced oxidative protein product (AOPP) levels. The potential beneficial effects of guarana on LDL levels and AOPP can provide a possible causal explanation for the lower prevalence of some cardiometabolic diseases in this population (KREWER *et al.,* 2011).

In fact, guarana has also shown to have antioxidant (BASILE *et al.,* 2005), antimicrobial (BASILE *et al.,* 2005), antimutagenic and anticarcinogenic effects (FUKUMASU, LATORRE and ZAIDAN-DAGLI, 2011). Some studies showed that guarana protects against testicular damage caused by cadmium exposition (LEITE *et al.,* 2012) and improves memory (KENNEDY *et al.,* 2004). On the other hand, some studies have shown low toxicity associated with guarana ingestion (BITTENCOURT *et al.,* 2013).

P. cupana extract from seeds is currently approved in the United States as a food additive for use as natural flavouring agent and is considered a dietary supplement under the DSHEA of 1994 (HEARD *et al.,* 2006). *P. cupana* is also listed in the official Brazilian Pharmacopoeia (ANTONELLI-USHIROBIRA *et al.,* 2010). Therefore, it is not surprising that *P. cupana* is found today in a wide variety of drinks, foods, dietary/herbal supplements and pharmaceuticals (LIMA *et al.,* 2005; ANTONELLI-USHIROBIRA *et al.,* 2010). Consequently, the HMPC of European Medicines Agency launched a call to encourage submission of scientific data on *P. cupana* (Guarana) in order to prepare the corresponding herbal monograph (EUROPEAN MEDICINES AGENCY, 2011). In the final document recently published was

established that persons taking monoamine oxidase (MAO) inhibitor drugs should use *P. cupana* seeds with caution and was established that caffeine-containing preparations reduce the sedative actions and increase the side effects caused by sympathomimetic drugs (EUROPEAN MEDICINES AGENCY, 2013).

I.7. *CITRUS AURANTIUM*

C. aurantium is the botanical name of a plant belonging to the family Rutaceae commonly known as bitter orange, sour orange, green orange, Seville orange, or zhi shi (BOUCHARD *et al.,* 2005; HAAZ *et al.,* 2006; HANSEN *et al.,* 2013). The extract of the immature fruit or peel of bitter orange has been used in traditional Chinese medicine for relief of indigestion, abdominal pain, constipation, and dysenteric diarrhea (HANSEN *et al.,* 2011). However, *C. aurantium* extract and its primary protoalkaloid *p*-synephrine are nowadays widely used in weight loss and thermogenic products (STOHS, PREUSS and SHARA, 2011a). In particular, after the sale of all ephedra-containing supplements were banned by FDA in 2004, the *C. aurantium* has gained an additional popularity as a safe alternative to *Ephedra* in herbal weight loss products (FOOD AND DRUG ADMINISTRATION and HHS, 2004; HANSEN *et al.,* 2012, 2013). A recent survey in Southeastern City found that *C. aurantium* was among the top ten ingredients (between herbal/botanical, vitamins, minerals, amino acids and proteins, animal or insect and others) in non-prescription weight loss supplements (SHARPE *et al.,* 2006). *C. aurantium* has been used in dietary supplements marketed for weight management products due to its claimed effects on metabolic processes, including an increase of the basal metabolic rate and lipolysis, and in appetite suppression (STOHS, PREUSS and SHARA, 2012).

The alkaloids *p*-synephrine, also called synephrine that constitutes about 85% of the protoalkaloids present in *C. aurantium*, and to a lesser extent octopamine are believed to be the most active components of *C. aurantium* (FUGH-BERMAN and MYERS, 2004; PEIXOTO *et al.,* 2012). *C. aurantium* peel contains other protoalkaloidal constituents including hordenine, tyramine and *N*-methyltyramine (STOHS, PREUSS and SHARA, 2011a). The extract also contains furanocoumarins and flavonoids, including limonene, hesperidin, neohesperidin, naringin, and tangaretin (FUGH-BERMAN and MYERS, 2004). The active components in *C. aurantium* are closely related to endogenous neurotransmitters (epinephrine and norepinephrine) and ephedrine (Figure I.7). However, despite the structural similarities in particular with ephedrine (one of the main active constituents in the genus Ephedra), synephrine seems to exhibit little or no stimulant activity on the cardiovascular system and central nervous system. Indeed, their chemical differences appear to significantly change their pharmacokinetic properties, particularly their ability to cross the blood-brain barrier (STOHS, PREUSS and SHARA, 2011b). Even so, the safety and efficacy of *C. aurantium* have been

questioned (BENT, PADULA and NEUHAUS, 2004; FUGH-BERMAN and MYERS, 2004; HANSEN *et al.,* 2013). In fact, a large number of case reports have associated the use of *C. aurantium*-containing products with several adverse effects most of them involving the cardiovascular system and some herb-drug interactions have been reported.

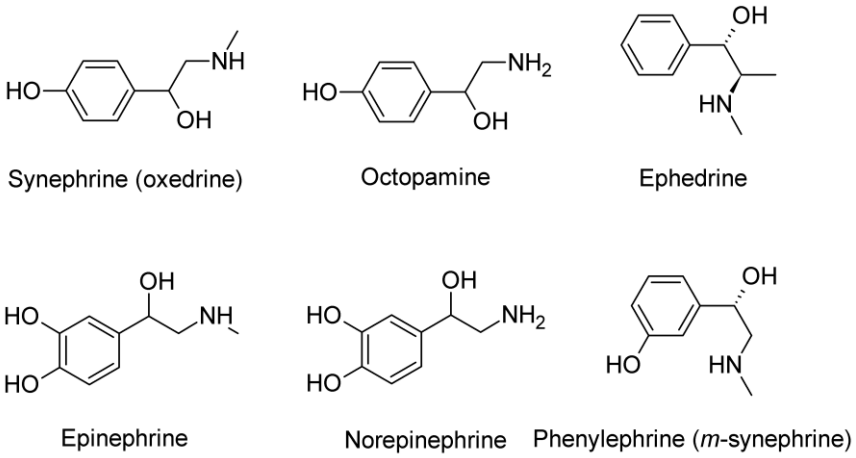


Figure I.7 – Chemical structures of synephrine, octopamine, ephedrine, epinephrine, norepinephrine and phenylephrine (FUGH-BERMAN and MYERS, 2004).

Due to the structural similarities, it has been assumed that synephrine has similar binding properties to that observed for norepinephrine and ephedrine and *m*-synephrine. However, according to receptor binding studies, synephrine exhibits low binding affinity for the α-, β-1 and β-2 adrenoreceptors. The binding to α-adrenergic receptors generally results in vasoconstriction, the binding to β-1 adrenergic receptors results in cardiovascular contractility and increase heart rate, and the binding to β-2 adrenergic receptors results in bronchodilation. On other hand, synephrine has shown β-3 adrenorecetpor activity resulting in increased thermogenesis and lipolysis (associated with the physiological action of the compound) (STOHS, PREUSS and SHARA, 2011a).

C. aurantium extracts marketed for weight-loss are prepared from dried fruit peels and may be associated with other compounds, mainly caffeine (BLANCK *et al.,* 2007). Despite its inclusion in over-the-counter weight loss products, little evidence supports the use of *C. aurantium* alone to treat overweight and obesity (HAAZ *et al.,* 2006). It has been shown that *C. aurantium* extracts increase lipolysis, thermogenesis and weight loss in both animal models and humans when tested in combination with other compounds, especially caffeine, which is also known to enhance the thermogenic properties of herbal weight-loss preparations (FUGH-BERMAN and MYERS, 2004; HAAZ *et al.,* 2006). As the *C. aurantium* extracts, synephrine has also effectively promoted weight-loss and thermogenesis in humans

and in other mammalian species only in combination with other stimulants (CARPÉNÉ *et al.,* 1999; HOFFMAN *et al.,* 2009; ROSSATO *et al.,* 2011), although in pure form it has been effective in mice and rats (CALAPAI *et al.,* 1999; ARBO *et al.,* 2009; STOHS, PREUSS and SHARA, 2011a).

I.8. *CARICA PAPAYA*

C. papaya, also known as papaya, papaw or pawpaw, is the fruit of a tree-like herbaceous plant belonging to the only species in the genus Carica of the plant family Caricaceae (MING, YU and MOORE, 2007; KRISHNA, PARIDHAVI and PATEL, 2008; ARAVIND *et al.,* 2013; THAM *et al.,* 2013). Papaya is cultivated in tropical and subtropical and temperate regions worldwide, including Australia, Brazil, China, Hawaii, Malaysia and India (CANINI *et al.,* 2007; AFZAN *et al.,* 2012). Papaya is a fast growing rarely branching, semi-woody tropical fruit tree with a short juvenile phase of 3 to 8 months. Once it starts flowering, it will continue to flower and produce fruit throughout the year (MING, YU and MOORE, 2007). Different parts of the plant (fruits, leaves, barks, roots, flowers, seeds, and latex) as well as some of their extracts have been traditionally used worldwide in folk medicine to treat a wide range of ailments in humans (ANUAR *et al.,* 2008; SADEK, 2012; NGUYEN *et al.,* 2013).

The fruits of *C. papaya* are one of the most commonly consumed throughout the world (ATHESH *et al.,* 2012), constituting a nutritional source rich in fibre, minerals and antioxidant nutrients (SADEK *et al.,* 2012). More specifically, papaya fruit is a good source of bioactive phytochemicals such as carotenoids (β-carotene, α-carotene, β-cryptoxanthin, α-cryptoxanthin, lutein, 9-*cis*-β-carotene), phenolic compounds (ferulic acid, caffeic acid, *p*-coumaric acid, rutin, quercetin, kaempferol), and glucosinolates (benzyl glucosinolate, benzyl isothiocyanate) (Figure I.8) (NGUYEN *et al.,* 2013). Unripe pulp of *C. papaya* also contains cardenolides which seem to have medicinal value for the treatment of congestive heart failure (ANUAR *et al.,* 2008). Additionally, among other biologically active constituents, papaya also includes cysteine proteinases as chymopapain and papain (KOVENDAN *et al.,* 2012; SADEK *et al.,* 2012; ARAVIND *et al.,* 2013). These two important proteolytic enzymes are widely known as being useful for the treatment of digestive disorders and disturbances of the gastrointestinal tract (SILVA *et al.,* 2007; ARAVIND *et al.,* 2013) and were found in the milky white latex extruded from fruits. The latex serves as an excellent meat tenderizer, for treatments of gangrenous wounds or burns, and is also used in cosmetic products, in the light industry and in food processing (SILVA *et al.,* 2007). Apart from papain and chymopapain, *C. papaya* also contains a lipase (CPL). CPL is tightly bonded to the water-insoluble fraction of crude papain and is thus considered as a "naturally immobilized" biocatalyst (DOMÍNGUEZ DE MARÍA *et al.,* 2006; SILVA *et al.,* 2007).

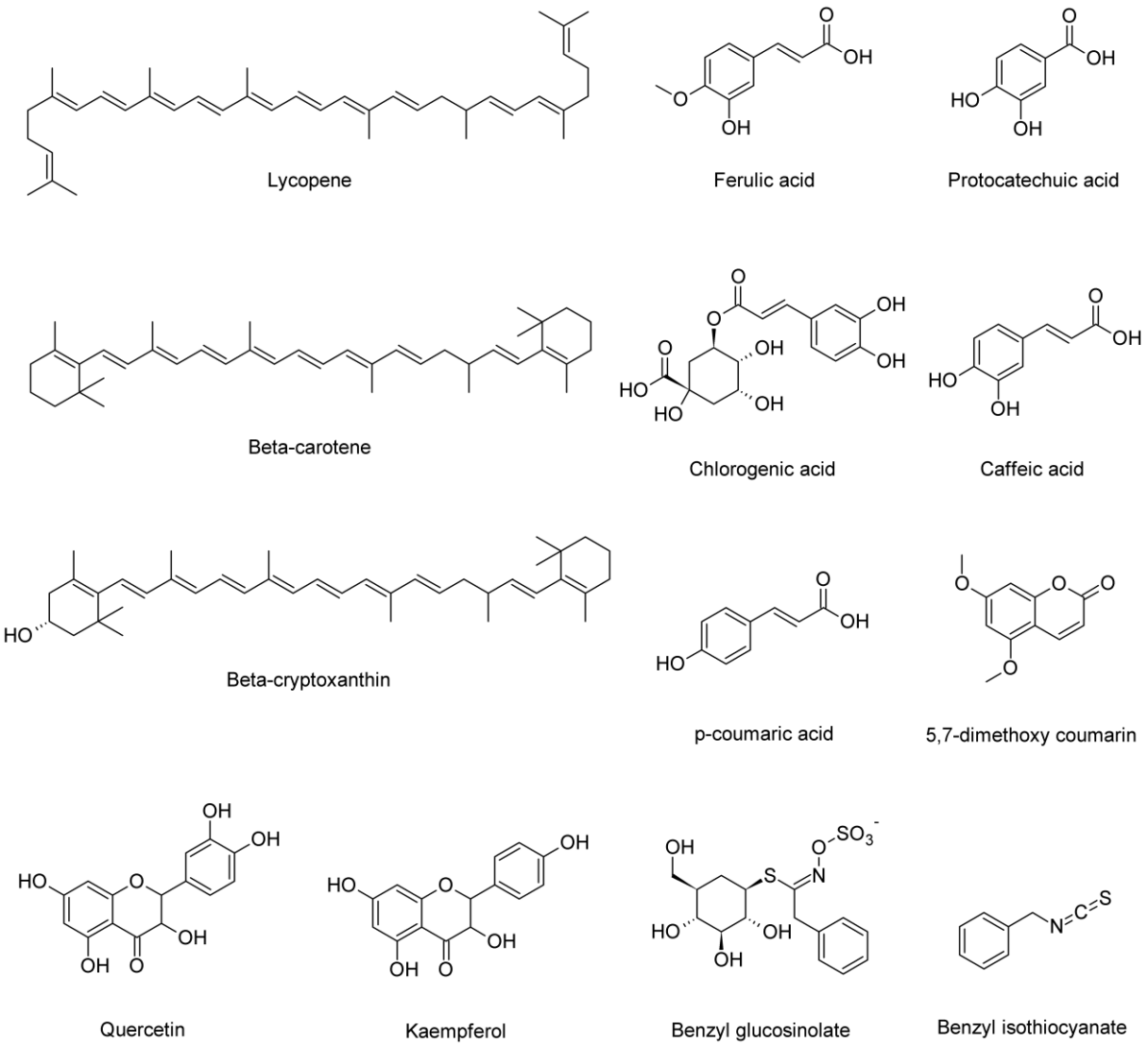


Figure I.8 – Chemical structures of important compounds found in *Carica papaya*: lycopene, ferulic acid, protocatechuic acid, beta-carotene, chlorogenic acid, caffeic acid, beta-cryptoxanthin, *p*-coumaric acid, 5,7-dimethoxy coumarin, quercetin, kaempferol, benzyl glucosinolate and benzyl isothiocyanate (NGUYEN *et al.,* 2013).

The nutrients of papaya as a whole improve cardiovascular system, protect against heart diseases, heart attacks, strokes and prevent colon cancer. The fruit prevents damage caused by free radicals that may cause some forms of cancer and is reported that it helps in the prevention of diabetic heart disease (ARAVIND *et al.,* 2013). Extracts of the ripe fruits are used for a variety of medicinal purposes ranging from treatment of ringworm, malaria, and hypertension (SADEK *et al.,* 2012), while the extracts of unripe fruit have been used in the treatment of ulcers (EZIKE *et al.,* 2009) and diabetes (SADEK *et al.,* 2012). In addition, the hypoglycemic and hypolipidemic effects of the aqueous seed extract of *C. papaya* have been

reported in rats (ADENEYE and OLAGUNJU, 2009). The ethanolic extract and water-soluble fraction of *C. papaya* also showed anti-hyperlipidemic activity in olive-treated rats (IVER *et al.,* 2011). More recently, ATHESH *et al.,* (2012) reported the anti-obesity effect of aqueous fruit extract of *C. papaya* in rats fed on high fat cafeteria diet. Effectively, besides the proteolytic enzymes, chymopapain and papain, CPL can contribute to its lipolytic action, supporting its use in the management of obesity and digestive disorders (IZZO, 2004; KOVENDAN *et al.,* 2012; SADEK *et al.,* 2012).

I.9. CARDIOVASCULAR DISEASES AND OBESITY: THE ROLE OF HERBAL MEDICINES

Despite the great progress achieved in the past century in the prevention and treatment of cardiovascular diseases, they remain the leading cause of mortality in industrialized countries at the beginning of the 21st century. Cardiovascular diseases continue to represent a major public health concern today. Epidemiological, pathophysiological and clinical studies have clearly indicated that overweight and obesity are major independent risk factors for coronary heart disease, atrial fibrillation and heart failure (SCAGLIONE *et al.,* 2004; ZALESIN *et al.,* 2011), and they have deleterious effects on ventricular function, cerebrovascular disease, peripheral arterial disease and venous thromboembolism (DOUKETIS and SHARMA, 2005). Thus, bearing in mind the increasing prevalence of obesity, reaching pandemic proportions in developed countries, an overall increase in the incidence of cardiovascular diseases is still expected over the next years (BODARY, IGLAY and EITZMAN, 2007). In consequence, it is also predictable the growing use of drugs currently available for the management of such cardiovascular diseases, existing among them drugs with a narrow therapeutic index such as AM (SINGH, 2008).

Taking into account the multifactorial nature of cardiovascular diseases, it emerges the need of multifaceted treatment strategies, focusing on the patient rather than in single risk factors. In all circumstances, to improve the cardiometabolic risk profile and to reduce the incidence of cardiovascular complications, body weight reduction has been established as an essential and also popular therapeutic strategy (LEE *et al.,* 2008; ALLCOCK, GARDNER and SOWERS, 2009). Hence, particularly worrying in developed countries is the increasing consumption of herbal weight loss medicines/dietary supplements as alternative or complement to the traditional programs of weight reduction. In addition, this issue is even more serious because among the general population prevails the notion that herbal preparations are devoid of health risks due to the natural origin of the constituents. Indeed, only a minority recognizes that herbal medicinal products are complex mixtures of phytochemicals, which may be substrates, inhibitors or inducers of the same biological systems that are involved in the biodisposition of conventional drugs, existing, therefore, a high potential for clinically significant pharmacokinetic interactions (CHAVEZ, JORDAN and CHAVEZ, 2006). Accordingly, considering the close relationship between obesity and cardiovascular diseases, as well as the scarcity of scientific information available about the safety of herbal slimming medicines/supplements, it is absolutely justified to assess the

potential for pharmacokinetic interactions between the most relevant herbal weight loss extracts and high-risk drugs required in multiple cardiovascular diseases such as the case of AM. Thus, from the perspective of protecting public health, it is warranted to characterize the interference of standardized herbal extracts commonly used in weight loss programs *(e.g. F. vesiculosus, P. cupana, C. aurantium* and *C. papaya)* on the pharmacokinetic profile of AM.

I.10. OBJECTIVES OF THIS THESIS

The main goal of this thesis was to carry out an *in vivo* preclinical systematic investigation to assess the potential for the occurrence of significant herb-drug interactions between commonly used standardized herbal weight loss extracts *(F. vesiculosus, P. cupana, C. aurantium and C. papaya)* and AM, a drug of narrow safety range widely used in clinical practice for the management of cardiovascular diseases.

Thus, an *in vivo* model (Wistar rat) was selected to study the pharmacokinetic interactions and an appropriate study design was adopted. Moreover, to obtain reliable data, it was necessary to develop and validate appropriate bioanalytical methods to quantify the analytes of interest in the biological matrices of the intended samples. In addition, a new bioanalytical tool was also develop to quantify AM and its main metabolite in human plasma, which may be useful to support the therapeutic drug monitoring of AM.

The specific objectives were outlined for the implementation of this work:

- Development and full validation of an analytical high-performance liquid chromatography method with diode array detection (HPLC-DAD) to simultaneously quantify AM and MDEA in human plasma using a sample preparation involving microextraction by packed sorbent (MEPS). The development of this HPLC technique enables to supply a new tool using an innovative sample preparation procedure to support the clinical monitoring of AM.

- Development and full validation of an analytical HPLC-DAD method to simultaneously quantify AM and MDEA in rat matrices (plasma and heart, liver, kidney and lung tissue homogenates). The development and validation of this technique in rat matrices enable to support the further herb-drug pharmacokinetic studies.

- Investigation of the interaction between each selected commercial standardized (certified) extracts (*F. vesiculosus*, *P. cupana*, *C. aurantium* or *C. papaya)* and AM in rats, following the simultaneous oral co-administration and after a 14-day pre-treatment period with each of the extracts.

- Evaluation of the effect of a 14-day pre-treatment period with each selected extract (*F. vesiculosus*, *P. cupana*, *C. aurantium* or *C. papaya)* on the body weight of the rats.

I.11. BIBLIOGRAPHY

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CHAPTER II

First Liquid Chromatographic Method for the Simultaneous Determination of Amiodarone and Desethylamiodarone in Human Plasma using Microextraction by Packed Sorbent (MEPS) as Sample Preparation Procedure

II. First Liquid Chromatographic Method for the Simultaneous Determination of Amiodarone and Desethylamiodarone in Human Plasma using Microextraction by Packed Sorbent (MEPS) as Sample Preparation Procedure

II.1. ABSTRACT

For the first time a simple and fast high-performance liquid chromatography (HPLC) method using a novel sample preparation procedure based on microextraction by packed sorbent (MEPS) was developed and validated for the determination of amiodarone (AM) and its main metabolite mono-*N*-desethylamiodarone (MDEA) in human plasma. Chromatographic separation of the analytes (AM and MDEA) and tamoxifen, used as internal standard (IS), was achieved within less than 5 min on a LiChroCART Purospher® Star C_{18} column (55 mm × 4 mm, 3 µm). The mobile phase consisting of 50 mM phosphate buffer with 0.1% formic acid (pH 3.1)/methanol/acetonitrile (45:5:50, v/v/v) was pumped isocratically at a flow rate of 1.2 mL/min. The detection was carried out at 254 nm. Calibration curves were linear ($r^2 \geq 0.9976$) in the ranges of 0.1-10 µg/mL for AM and MDEA. The limits of quantification were established at 0.1 µg/mL for AM and MDEA. The overall imprecision did not exceed 6.67% and inaccuracy was within ±9.84%. The overall mean recovery of AM and MDEA ranged from 58.6% to 68.2%. Neither endogenous nor tested exogenous compounds were found to interfere at retention times of the analytes (AM and MDEA) and IS. This new MEPS/HPLC method was also applied to real samples obtained from polymedicated patients receiving AM therapy. Thus, this bioanalytical method seems to be a useful tool for therapeutic drug monitoring of patients under AM treatment and also to support other clinical pharmacokinetic-based studies involving this drug, such as bioavailability/bioequivalence studies.

II.2. INTRODUCTION

Amiodarone [2-*n*-butyl-3-(3,5-diiodo-4-diethylaminoethoxy-benzoyl)-benzofuran; AM] (Figure II.1) is one of the most frequently prescribed antiarrhythmic drugs despite the availability of novel antiarrhythmic agents (WOLKOVE and BALTZAN, 2009; PAPIRIS *et al.,* 2010). Nevertheless, the unusual pharmacokinetic properties of AM and its primary metabolite mono-*N*-desethylamiodarone (MDEA; Figure II.1) complicate the clinical use of the drug. Actually, the high lipid solubility of AM and MDEA leads to an extensive and variable accumulation of both compounds in different tissues, resulting in a marked delay in onset of pharmacological action and in a number of safety concerns particularly after several weeks to months of AM therapy (MCKENNA, ROWLAND and KRIKLER, 1983; VERONESE, MCLEAN and HENDRIKS, 1988; CAMPBELL and WILLIAMS, 2001; WOLKOVE and BALTZAN, 2009). Therefore, the long-term use of AM is limited by serious or even life-threatening adverse events, such as thyroid dysfunction, pulmonary toxicity and hepatic toxicity (SEKI *et al.,* 2008; LAFUENTE-LAFUENTE *et al.,* 2009; WOLKOVE and BALTZAN, 2009).

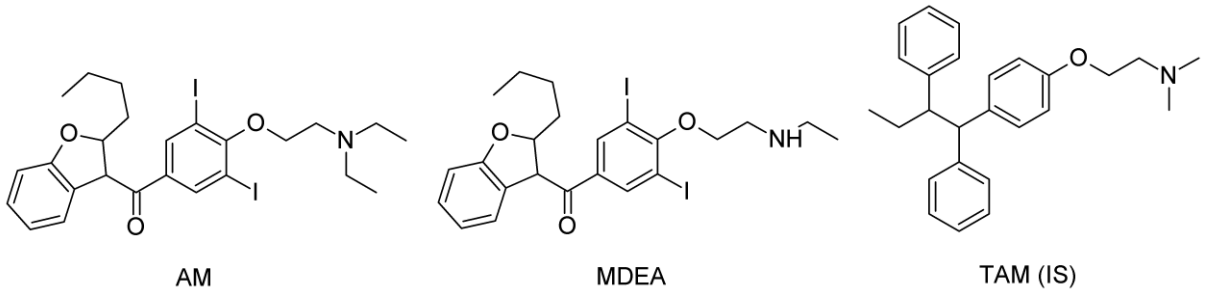

Figure II.1 – Chemical structures of amiodarone (AM), mono-*N*-desethylamiodarone (MDEA) and tamoxifen (TAM) used as internal standard (IS).

Indeed, AM has long been recognised as a drug having a narrow therapeutic window (0.5-2.0 µg/mL) (STÄUBLI *et al.,* 1983; LESNE and PELLEGRIN, 1987; POLLAK, BOUILLON and SHAFER, 2000; PÉREZ-RUIZ *et al.,* 2002; SHAYEGANPOUR, HAMDY and BROCKS, 2008). Several studies have reported that serious toxicity is more likely at AM serum/plasma concentrations above 2.5 µg/mL (ROTMENSCH *et al.,* 1984; POLLAK and SHAFER, 2004); ROTMENSCH *et al.* (1983) also concluded that AM serum concentrations below 2.5 µg/mL significantly improve AM's benefit-to-risk relationship. Furthermore, the therapeutic drug monitoring (TDM) of AM plasma concentrations may differentiate treatment failure from suboptimal dosing and may reduce the incidence of concentration-related adverse effects

(ROTMENSCH *et al.,* 1984). On the other hand, the monitoring of AM and MDEA plasma concentrations may be of particular interest to compare different routes of administration (KOTAKE *et al.,* 2006) and to assess the impact of the switching in drug formulation (innovator *versus* generic medicine) (SAURO *et al.,* 2002). Therefore, AM can be used more safely when the clinician is aware of the concentrations the patient is exposed to and adjusts the dose as necessary. For that, the availability of a fast, sufficiently sensitive, and selective bioanalytical method to enable the reliable determination of AM and its pharmacologically active metabolite (MDEA) is required.

Until now, a lot of bioanalytical methods have been reported in literature for the determination of AM and MDEA in human plasma and other biological fluids. However, in those methods the sample preparation has been carried out by means of liquid-liquid extraction (LLE) (STOREY and HOLT, 1982; GUPTA and CONNOLLY, 1984; MARCHISET *et al.,* 1984; SHIPE, 1984; HUTCHINGS, SPRAGG and ROUTLEDGE, 1986; LESNE and PELLEGRIN, 1987; DE SMET and MASSART, 1988; PACZKOWSKI *et al.,* 1989; ROCHAS *et al.,* 1989; JUENKE *et al.,* 2004), solid-phase extraction (SPE) (SUSANTO, HUMFELD and REINAUER, 1986; VERBESSELT, TJANDRAMAGA and DE SCHEPPER 1991; KOLLROSER and SCHOBER, 2002; KUHN, GOTTING and KLEESIEK, 2010), protein precipitation (PP) (PLOMP *et al.,* 1983; WEIR and UEDA, 1985; RAJENDRAN *et al.,* 2006) and combining different procedures such as PP and LLE (GUMIENICZEK, MISZTAL and PRZYBOROWSKI, 1994; BOLDERMAN, HERMANS and MAESSEN, 2009). Nevertheless, the recent developments in the field of sample preparation have been directed toward miniaturization and automation, and one of the latest developments was the emergence of microextraction by packed sorbent (MEPS), which was already used for the quantitative analysis of several drugs such as antidepressants (CHAVES *et al.,* 2010), methadone (EL-BEQQALI and ABDEL-REHIM, 2007), acebutolol and metoprolol (EL-BEQQALI *et al.,* 2007), atorvastatin (VLČKOVÁ *et al.,* 2011), pravastatin (VLČKOVÁ *et al.,* 2012), oxcarbazepine (SARACINO, TALLARICO and RAGGI, 2010), risperidone (SARACINO *et al.,* 2010; MANDRIOLI *et al.,* 2011) and remifentanil (SAID *et al.,* 2011), among others. This novel approach for sample preparation is essentially a miniaturized version of SPE using 1-4 mg of sorbent packed either inside a syringe (100-250 µL) as a plug or between the barrel and the needle as a cartridge (ABDEL-REHIM, 2010, 2011). In fact, there is a constant need for the development of faster and more selective sample clean-up procedures and MEPS represents a new approach suitable for the rapid analysis of drugs and/or metabolites from biological

fluids. Nevertheless, to the best of our knowledge none bioanalytical assay was previously developed for the determination of AM using MEPS as sample preparation procedure.

Thus, the aim of this work was to develop and fully validate, for the first time, a high-performance liquid chromatography (HPLC) method for the simultaneous quantification of AM and MDEA in human plasma using the innovative MEPS technology for sample preparation.

II.3. MATERIALS AND METHODS

II.3.1 CHEMICALS AND REAGENTS

AM (lot no. 078K1246) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and MDEA (lot no. LB33020) was kindly supplied by Sanofi-Aventis (Paris, France). Tamoxifen as citrate salt (TAM; lot no. 035K1270) was obtained from Sigma-Aldrich (St Louis, MO, USA) and it was used as internal standard (IS). The chemical structures of these compounds are shown in Figure II.1. Methanol (HPLC grade) and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Leicestershire, United Kingdom). Ultra-pure water (HPLC grade, >18 MΩ) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). All other reagents were of analytical grade: sodium phosphate monobasic purris p.a. (Sigma-Aldrich GmbH; Seelze, Germany), sodium phosphate dibasic anhydrous (Sigma-Aldrich; St Louis, MO, USA), sodium dihydrogen phosphate dehydrate purum p.a. (Fluka Chemie; Buchs, Switzerland) and formic acid (98-100%) (Merck KGaA; Darmstadt, Germany). MEPS 100 µL syringe and MEPS BIN (barrel insert and needle) containing ~4 mg of solid-phase silica - C_{18} material (SGE Analytical Science, Australia) were purchased from ILC (Porto, Portugal). Blank human plasma from healthy blood donors was kindly provided by the Portuguese Blood Institute after the written consent of each subject and in accordance with the principles of Helsinki Declaration.

II.3.2 STOCK SOLUTIONS, CALIBRATION STANDARDS AND QUALITY CONTROL SAMPLES

Stock solutions of AM, MDEA, and TAM (IS) at the concentration of 1 mg/mL were individually prepared by dissolving appropriate amounts of each compound in methanol. Appropriate volumes of each of the stock solutions of AM and MDEA were combined and diluted in methanol to obtain an intermediate solution at 100 µg/mL. Thereafter, stock and intermediate solutions were appropriately used to afford six combined spiking solutions at final concentrations of 0.5, 1, 2.5, 7.5, 20 and 50 µg/mL for AM and MDEA, which were used to spike blank human plasma in order to prepare plasma calibration standards at six different concentration levels: 0.1, 0.2, 0.5, 1.5, 4 and 10 µg/mL for AM and MDEA. The stock solution of IS was daily diluted with water-methanol (60:40, v/v) in order to obtain a working solution

of 25 µg/mL. All solutions were stored protected from light at approximately 4 °C for one month, except the IS working solution which was prepared daily.

Quality control (QC) samples were prepared independently in the same matrix (blank human plasma), at three different concentration levels, representing the low (QC_1), middle (QC_2) and high (QC_3) ranges of the calibration curves. Aliquots of blank human plasma were appropriately spiked to achieve the concentrations for both analytes (AM and MDEA) of 0.3 µg/mL in QC_1; 5 µg/mL in QC_2 and 9 µg/mL in QC_3.

II.3.3 APPARATUS AND CHROMATOGRAPHIC CONDITIONS

Chromatographic analysis was carried out using an HPLC system (Agilent 1260 Infinity Quaternary LC system) coupled with a diode-array detector (Agilent 1260 Infinity; G1315D DAD VL). All instrumental parts were automatically controlled by Agilent ChemStation software (Agilent Technologies).

The chromatographic separation of AM, MDEA and IS was achieved in less than 5 min and it was carried out at room temperature, by isocratic elution with a mobile phase of 50 mM phosphate buffer with 0.1% formic acid (pH 3.1)/methanol/acetonitrile (45:5:50, v/v/v), at a flow-rate of 1.2 mL/min, on a reversed-phase LiChroCART® Purospher Star-C_{18} column (55 mm × 4 mm; 3 µm particle size) purchased from Merck KGaA (Darmstadt, Germany). The mobile phase was filtered through a 0.45 µm filter and degassed ultrasonically for 15 min before use. The injection volume was 20 µL and the wavelength of 254 nm was selected for the detection of all compounds (AM, MDEA and IS).

II.3.4 SAMPLE PREPARATION AND EXTRACTION

The sample preparation was previously optimized and the final conditions were as follows. Each aliquot (100 µL) of human plasma samples, spiked with 20 µL (0.5 µg) of the IS working solution, was added of 300 µL of ice-cold acetonitrile. The mixture was vortex-mixed for 30 s and centrifuged at 17000 rpm for 2 min at 4 °C. Afterwards, the supernatant was transferred to a clean vial, diluted with 400 µL of ultra-pure water and this mixture volume was then submitted to MEPS procedure. Briefly, the MEPS sorbent (C_{18}) was manually conditioned with 3 × 100 µL of methanol/formic acid (95:5, v/v) followed by

2 × 100 µL of ultra-pure water. After that, the whole volume of the diluted supernatant was drawn through the sorbent and ejected at a flow rate of approximately 10 µL/s (this procedure was performed twice). The sorbent was washed with 100 µL of ultra-pure water in order to remove interferences, and then the analytes were eluted with 100 µL of methanol/formic acid (95:5, v/v). An aliquot (20 µL) of the eluted sample was injected into the chromatographic system. To avoid the carryover the MEPS sorbent was sequentially washed/reconditioned with 30 × 100 µL of methanol/formic acid (95:5, v/v) and 2 × 100 µL of ultra-pure water before the application of following sample. The carryover was carefully investigate on MEPS sorbent and no effect was evident using the previously referred conditions after the extraction and analysis of successive aliquots at the highest standard concentrations followed by extraction and analysis of aliquots of blank plasma. Each MEPS device was re-used in about 100 extraction cycles before being discarded.

II.3.5 METHOD VALIDATION

The described method was validated according to internationally accepted recommendations for bioanalytical method validation (SHAH *et al.,* 2000; GUIDANCE FOR INDUSTRY, 2001; EUROPEAN MEDICINES AGENCY, 2011).

Selectivity was evaluated by analysing blank plasma samples from six different sources to ensure the absence of chromatographic interferences from endogenous compounds (matrix effects) at the retention times of MDEA, AM and IS. In addition, interferences from other drugs usually co-administered with AM in clinical practice were also evaluated injecting, under the optimized chromatographic conditions, standard solutions of these compounds at a concentration of 10 µg/mL.

To evaluate the linearity of the analytical method, calibration curves were prepared using six calibration standards in the range of 0.1-10 µg/mL and assayed on five different days (n = 5). The calibration curves were constructed by plotting analytes (MDEA or AM)/IS peak area ratios as function of the corresponding nominal concentrations. The data were subjected to a weighted linear regression analysis (ALMEIDA, CASTEL-BRANCO and FALCÃO, 2002).

The limit of quantification (LOQ) was defined as the lowest concentration of the calibration curve that can be measured with acceptable inter and intraday precision and accuracy, which were assessed respectively by the coefficient of variation (CV) not

exceeding 20% and the deviation from nominal concentration value (*bias*) within ±20%. The LOQs for MDEA and AM were evaluated by analysing plasma samples which were prepared in five replicates (n = 5). The limit of detection (LOD) of the analytes, defined as the lowest concentration that can be distinguished from the noise level, was established as the concentration that yields a signal-to-noise ratio of 3:1.

Intra and interday precision and accuracy were assessed by using plasma QC samples analysed in replicate (n = 5) at three concentration levels (low, medium and high QC samples) representative of the calibration range. The concentrations to be tested were 0.3, 5 and 9 μg/mL for MDEA and AM. The acceptance criterion for intra and interday precision (expressed as percentage of CV) was a CV value equal to or lower than 15% (or 20% in the LOQ) and for accuracy (expressed as percentage of *bias*) was a *bias* value within ±15% (or ±20% in the LOQ).

The recovery of the analytes from human plasma samples was calculated using three QC samples and the procedure described in the *section II.3.4*. The recoveries of the analytes (MDEA and AM) were calculated by comparing the analytes peak area from extracted samples against the corresponding areas obtained by direct injection of solutions at the same theoretical concentrations. The recovery of the IS was determined at the concentration used in sample analysis by calculating the peak area ratio of the IS in extracted samples and non-extracted solutions.

Human plasma stability of AM and MDEA was assessed at low (QC_1) and high (QC_3) concentration levels, in replicate (n = 5), at room temperature for 4 h, at 4 °C for 24 h, at −20 °C for 30 days, and at −80 °C for 30 days to simulate sample handling and storage time in the freezer before analysis. The stability of AM and MDEA was also studied at 4 °C during 24 h in the processed samples (post-preparative stability) to simulate the time that samples can be in the auto-sampler before analysis. The effect of three freeze-thaw cycles on the stability of the analytes (AM and MDEA) was also investigated in plasma at −20 °C and −80 °C. Aliquots of spiked plasma samples (QC_1 and QC_3) were stored at −20 °C and at −80 °C for 24 h, thawed unassisted at room temperature, and when completely thawed samples were refrozen for 24 h under the same conditions until completing the three cycles. Stability was assessed comparing the data of QC samples analysed before (reference samples) and after being exposed to the conditions for stability assessment (stability samples); a stability/reference samples ratio of 85-115% was accepted as stability criterion (n = 5).

II.3.6 METHOD APPLICATION TO THE ANALYSIS OF REAL PLASMA SAMPLES

To evaluate the validity of the proposed bioanalytical method and its high selectivity for clinical application a set of real plasma samples obtained from ten polymedicated adult patients (5 males and 5 females) admitted at the Coimbra University Hospital and having in common the treatment with AM (200 mg tablets administered *per os* at different dosing regimens: once a day, twice a day or five days a week) were analysed. The blood samples were taken at the morning (7 am) and the informed consent was obtained from each subject. The analysis of these samples also represents an important approach to re-assess the selectivity of the developed method. Indeed, the number of different drugs co-prescribed with AM ranged from 6 (ID_2, ID_3 and ID_5) to 19 (ID_{10}) considering individually the patients, and were sixty-two different active pharmaceutical ingredients taking simultaneously all patients into account (ID_1-ID_{10}) (Table II.1).

Table II.1 – Range of drugs co-prescribed with amiodarone in ten hospitalized patients from which real plasma samples were collected and analysed using the HPLC method described.

Patients	Drugs
ID_1	Acetylcysteine, Allopurinol, Aminophylline, **Amiodarone**, Darbepoetin Alfa, Digoxin, Enoxaparin Sodium, Folic Acid, Furosemide, Metolazone, Oxazepam, Pantoprazole, Paracetamol, Piperacillin/Tazobactam, Spironolactone
ID_2	**Amiodarone**, Ceftazidime, Dosulepine, Fluconazole, Lactulose, Lysine Acetylsalicylate, Omeprazole
ID_3	**Amiodarone**, Ciprofloxacin, Diosmin, Finasteride, Furosemide, Ramipril, Warfarin
ID_4	**Amiodarone**, Azithromycin, Digoxin, Enoxaparin Sodium, Furosemide, Nitroglycerin, Omeprazole, Oxazepam, Piperacillin/Tazobactam, Spironolactone, Warfarin
ID_5	**Amiodarone**, Ampicillin, Fentanyl, Lactulose, Metoclopramide, Nystatin, Ranitidine
ID6	Acetylcysteine, **Amiodarone**, Bromide Ipatropium, Captopril, Ceftazidime, Folic Acid, Furosemide, Insulin, Levodopa/Carbidopa, Lorazepam, Metronidazole, Omeprazole, Paracetamol, Tramadol
ID_7	Acetylcysteine, Acetylsalicylic Acid, **Amiodarone**, Atorvastatin, Bromide Ipatropium/Salbutamol, Ciprofloxacin, Digoxin, Enoxaparin Sodium, Flavoxate, Furosemide, Oxazepam, Pantoprazole, Paracetamol, Spironolactone
ID_8	Acetylcysteine, **Amiodarone**, Bromazepam, Digoxin, Enalapril Maleate, Enoxaparin Sodium, Furosemide, Pantoprazole, Sertraline, Simvastatin, Spironolactone
ID_9	Acetylcysteine, Alprazolam, **Amiodarone**, Atorvastatin, Carvedilol, Digoxin, Enalapril Maleate, Enoxaparin Sodium, Furosemide, Pantoprazole, Propylthiouracil, Spironolactone, Triflusal
ID_{10}	Acetylcysteine, Allopurinol, Aminophylline, **Amiodarone**, Bromide Ipatropium/Salbutamol, Bromocriptine, Dopamine, Furosemide, Levofloxacin, Melperone, Meropenem, Metoclopramide, Metolazone, Morphine, Oxazepam, Pantoprazole, Paracetamol, Sertraline, Sucralfate, Zolpidem

ID, individual.

II.4. RESULTS

II.4.1 METHOD VALIDATION

II.4.1.1 Selectivity

The analysis of blank human plasma samples showed no endogenous interferences at the retention times of the compounds of interest (IS, MDEA and AM). Typical chromatograms of the extracts obtained from blank and spiked human plasma samples are shown in Figure II.2. Likewise, none of the tested drugs commonly co-prescribed with AM were found to interfere at the retention times of the chromatographic peaks of IS, MDEA and AM (Table II.2).

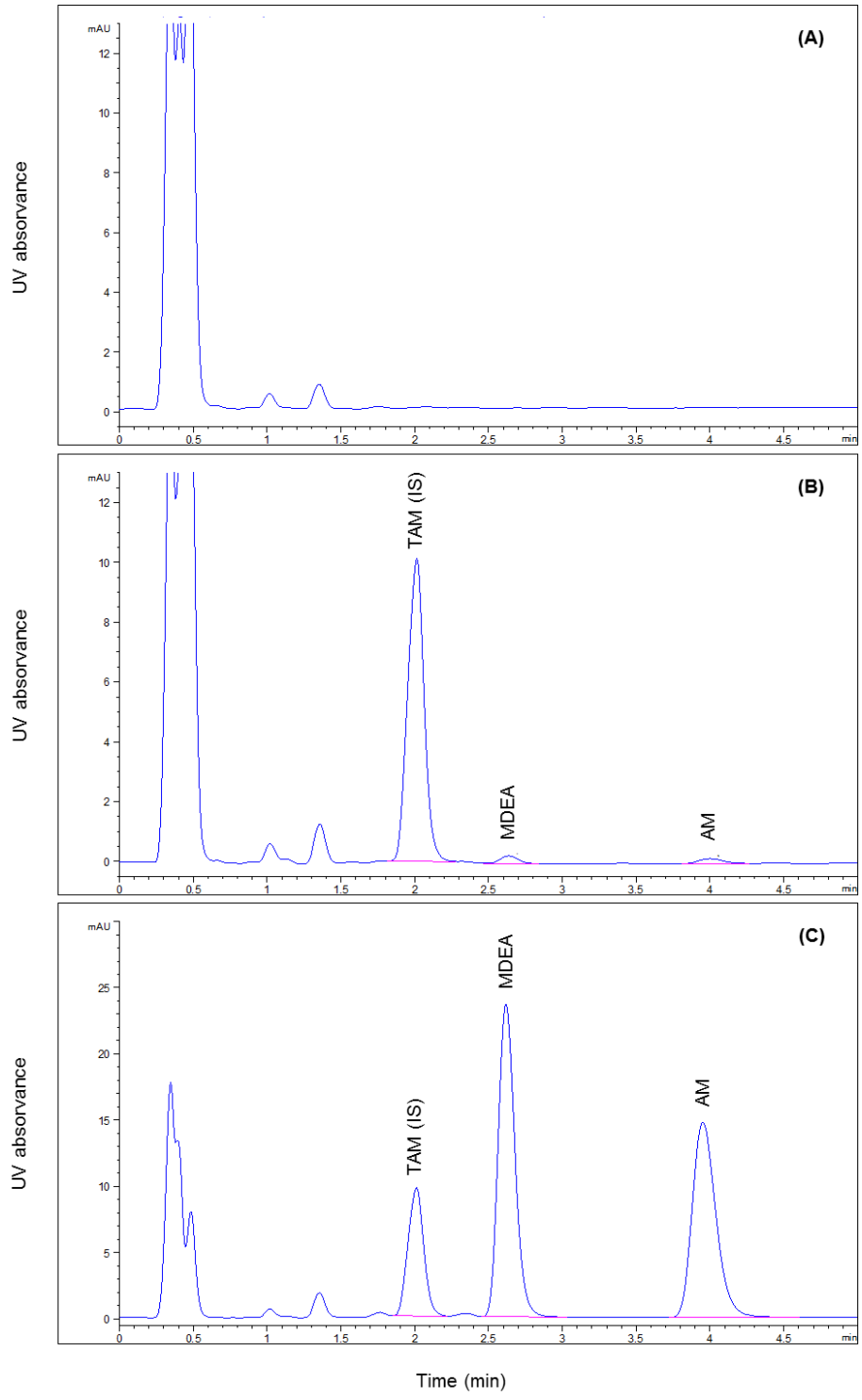

Figure II.2 – Typical chromatograms of extracted human plasma samples obtained by the MEPS/HPLC method developed: blank plasma (A); plasma spiked with internal standard [IS; tamoxifen (TAM)] and the analytes [mono-*N*-desethylamiodarone (MDEA) and amiodarone (AM)] at concentrations of the limit of quantification (0.1 μg/mL) (B) and at concentrations of the upper limit of calibration range (10 μg/mL) (C).

Table II.2 – Retention times of some drugs potentially co-prescribed with amiodarone (AM) examined as possible exogenous interferences.

Drugs	RT (min)	Drugs	RT (min)
Analgesics/Antipyretics		*Antiepileptics (continuation)*	
Acetylsalicylic acid	0.53	Carbamazepine	0.68
Paracetamol	0.40	Phenytoin	0.66
Nimesulide	1.43	Topiramate	ND
Antiarrhythmics		Phenobarbital	0.57
Flecainide	0.52	*Antihypertensives*	
Bepridil	1.59	Propranolol	0.45
Verapamil	0.57	Amiloride	0.34
Diltiazem	0.49	Nifedipine	1.15
Adenosine	0.32	Candesartan	8.28
Anticoagulants		*Antipsychotics*	
Warfarin	1.40	Chlorpromazine	0.72
Antidepressants		Haloperidol	0.57
Fluoxetine	0.65	Droperidol	0.44
Sertraline	0.72	*Sedatives/Hypnotics*	
Imipramine	0.77	Alprazolam	0.76
Trazodone	0.42	Clobazam	1.06
Maprotiline	0.61	Diazepam	1.46
Antiepileptics		Zolpidem	0.42
Lamotrigine	0.39	Promethazine	0.54

RT, retention time; ND, not detected within 20 min after the chromatographic injection.

II.4.1.2 Calibration curves, limits of quantification and limits of detection

The calibration curves obtained in human plasma for MDEA and AM were linear ($r^2 \geq 0.9976$) over the concentration range of 0.1-10 µg/mL. Due to the wide calibration range established, and in order to counteract the heteroscedasticity detected, the use of weighted linear regression analysis was required. The calibration curves were subjected to weighted linear regression analysis using $1/x^2$ as the weighting factor. Indeed, taking into account the plots and the sums of absolute percentage relative error as statistical criteria, the best-fit weighting factor for both compounds (AM and MDEA) was shown to be $1/x^2$ between the

weighting factors usually tested under heteroscedasticity conditions ($1/\sqrt{x}$, $1/x$, $1/x^2$, $1/\sqrt{y}$, $1/y$ and $1/y^2$). The weighted regression equations (n = 5) of the calibration curves were $y = 0.000238x - 0.000325$ ($r^2 = 0.9987$) for MDEA and $y = 0.000222x + 0.000356$ ($r^2 = 0.9976$) for AM, where y represents the analyte/IS peak area ratio and x represents the plasma concentration. The LOQs of the method were set at 0.1 µg/mL for MDEA and AM, with good precision (CV ≤ 6.67%) and accuracy (*bias* ± 5.29%) (Table II.3). The LODs were established at 0.02 µg/mL for MDEA and AM.

II.4.1.3 Precision and accuracy

The data for intra and interday precision and accuracy obtained from QC plasma samples at three different concentration levels (QC_1, QC_2 and QC_3) are shown in Table II.3. The intra and interday CV values did not exceed 5.16%, and the intra and interday *bias* values varied between −9.84 and 0.78%.

Table II.3 – Precision (% CV) and accuracy (% *bias*) for the determination of mono-*N*-desethylamiodarone (MDEA) and amiodarone (AM) in human plasma samples at the concentrations of the limit of quantification (*) and at the low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration ranges (n = 5).

Analyte	$C_{nominal}$ (µg/mL)	Precision (% CV)	Accuracy (% *bias*)
Interday			
MDEA	0.1*	2.34	4.73
	0.3	5.16	−3.96
	5	3.46	0.78
	9	2.51	−0.09
AM	0.1*	4.20	5.21
	0.3	2.09	−9.84
	5	2.55	−7.51
	9	2.74	−6.01

Table II.3 – Precision (% CV) and accuracy (% *bias*) for the determination of mono-*N*-desethylamiodarone (MDEA) and amiodarone (AM) in human plasma samples at the concentrations of the limit of quantification (*) and at the low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration ranges (n = 5) *(cont.).*

Analyte	$C_{nominal}$ (µg/mL)	Precision (% CV)	Accuracy (% *bias*)
Intraday			
MDEA	0.1*	3.39	5.21
	0.3	4.29	0.20
	5	0.94	−0.57
	9	1.06	−1.47
AM	0.1*	6.67	5.29
	0.3	2.53	−4.26
	5	2.63	−7.53
	9	1.44	−6.73

$C_{nominal}$, nominal concentration; CV, coefficient of variation.

II.4.1.4 Recovery

The recovery of MDEA and AM from human plasma samples spiked at three different concentration levels (QC_1, QC_2 and QC_3) was evaluated and the results are presented in Table II.4. The mean recoveries of MDEA and AM ranged from 64.7 to 68.2% and 58.6 to 62.3% respectively, and showed low CV values. The mean recovery of the IS was 61.0%, with a CV of 9.51%.

Table II.4 – Recovery (%) of mono-*N*-desethylamiodarone (MDEA) and amiodarone (AM) from human plasma samples at the low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration ranges (n = 5).

Analyte	$C_{nominal}$ (µg/mL)	Recovery (%)	
		Mean ± SD	CV (%)
MDEA	0.3	65.9 ± 5.38	8.17
	5	64.7 ± 0.83	1.28
	9	68.2 ± 1.50	2.19
AM	0.3	59.7 ± 4.71	7.89
	5	58.6 ± 2.12	3.62
	9	62.3 ± 1.73	2.78

$C_{nominal}$, nominal concentration; SD, standard deviation; CV, coefficient of variation.

II.4.1.5 Stability

The stability of MDEA and AM in human plasma was evaluated under different circumstances, simulating the handling and sample storage conditions likely to be encountered during the analytical process, by analysing low and high QC samples in replicate (n=5). The results of the stability assays showed that no significant loss was observed for AM and MDEA in human plasma at room temperature for 4 h, at 4 °C for 24 h, and at −20 °C and −80 °C for 30 days. The analytes also demonstrated to be stable in processed plasma samples at 4 °C during 24 h. The stability data for MDEA and AM evaluated after three freeze-thaw cycles at −20 °C and −80 °C indicated that the stability criteria previously established were not fulfilled only at −20 °C for QC_3. The stability data are shown in Table II.5.

Table II.5 – Stability (values in percentage) of mono-*N*-desethylamiodarone (MDEA) and amiodarone (AM) in unprocessed plasma samples at room temperature for 4 h, at 4 °C for 24 h, after three freeze-thaw cycles (−20 °C and −80 °C), and at -20 °C and −80 °C for 30 days; and in processed plasma samples left at 4 °C for 24 h (n = 5).

Analyte	MDEA		AM	
$C_{nominal}$ (μg/mL)	0.3	9	0.3	9
Unprocessed plasma				
Room temperature (4 h)	94.4	94.9	93.6	93.7
4 °C (24 h)	91.6	91.9	96.1	90.3
Freeze-thaw (3 cycles; −20 °C)	86.0	81.3	91.5	84.6
Freeze-thaw (3 cycles; −80 °C)	97.0	89.1	103.3	93.1
−20 °C (30 days)	97.7	96.4	103.0	101.5
−80 °C (30 days)	97.2	90.8	96.6	97.5
Processed plasma				
4 °C (24 h)	96.6	104.2	98.4	108.5

$C_{nominal}$, nominal concentration.

II.4.2 ANALYSIS OF REAL PLASMA SAMPLES

The MEPS/HPLC method was applied to the analysis of MDEA and AM in plasma samples taken from ten polymedicated patients orally treated with AM and the concentrations obtained are summarized in Table II.6, as well as the prescribed AM regimens. In addition, a representative chromatogram (ID_{10}) of the analyses of such real plasma samples is also depicted in Figure II.3. As one can see, no interference from human plasma endogenous compounds or the co-administered drugs is apparent, and it is also clearly seen that the peak shape of the analytes (IS, MDEA and AM) and chromatographic resolution are similar to those obtained after the analysis of spiked human plasma samples.

Table II.6 – Plasma concentrations of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) in real plasma samples obtained from polymedicated patients taking AM orally (200 mg tablets) at different prescribed regimens. All samples were collected at the morning (7 am).

Patients	Prescribed regimen	$C_{measured}$ (μg/mL)	
		AM	MDEA
ID_1	200 mg tablet (bid) / (9 am; 9 pm)	0.333	0.165
ID_2	200 mg tablet (5 days a week) / (7 pm)	0.622	0.234
ID_3	200 mg tablet (id) / (9 am)	BLQ	BLQ
ID_4	200 mg tablet (id) / (9 am)	0.129	BLQ
ID_5	200 mg tablet (5 days a week) / (7 pm)	0.512	0.236
ID_6	200 mg tablet (id) / (9 am)	0.122	0.118
ID_7	200 mg tablet (id) / (9 am)	0.124	BLQ
ID_8	200 mg tablet (id) / (9 am)	0.858	0.624
ID_9	200 mg tablet (id) / (9 am)	0.664	0.408
ID_{10}	200 mg tablet (id) / (9 am)	0.560	0.644

ID, individual; $C_{measured}$, measured concentration; bid, twice a day; id, once a day; BLQ, below the limit of quantification (< 0.1 μg/mL).

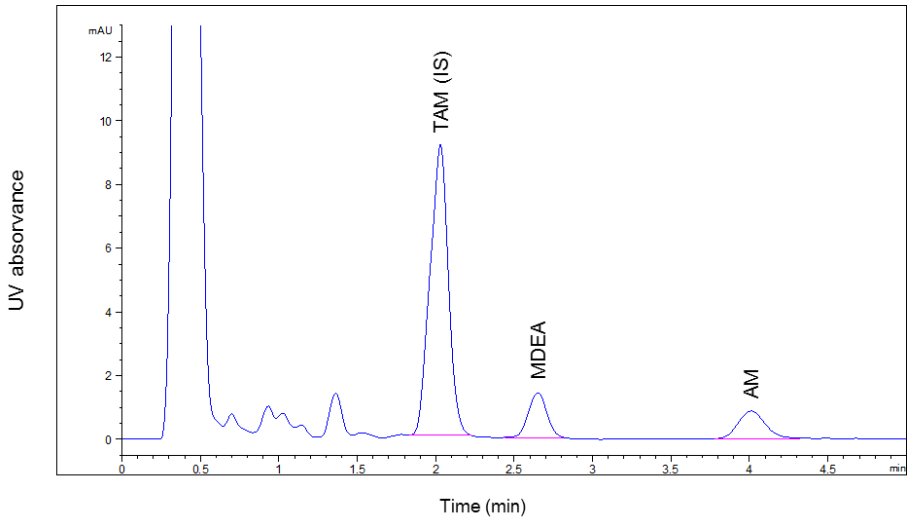

Figure II.3 – Representative chromatogram of the analysis of real plasma samples obtained from polymedicated patients under treatment with amiodarone (AM). Specifically, this chromatogram was generated by the analysis of the sample collected from patient ID_{10}, which was also the most polymedicated patient; the plasma concentrations of mono-*N*-desethylamiodarone (MDEA) and AM measured in this sample were respectively 0.644 μg/mL and 0.560 μg/mL.

II.5. DISCUSSION

For the first time a simple, fast and reliable MEPS/HPLC method was developed and fully validated for the simultaneous determination of AM and its main metabolite (MDEA) in human plasma. The optimized MEPS procedure enabled the validation of the method for the quantitative analysis of AM and MDEA within a wide concentration range (0.1-10 µg/mL), which includes the therapeutic window usually proposed for AM (0.5-2.0 µg/mL), and provided an acceptable extraction recovery (~60%) for all compounds of interest [TAM (IS), MDEA and AM].

The MEPS procedure developed presents several advantages in comparison with the sample preparation techniques usually used in bioanalysis. Firstly, the MEPS procedure does not require the evaporation and reconstitution steps of the sample usually needed in most of the LLE or SPE techniques. In addition, after the initial protein precipitation step, the MEPS procedure herein developed could be automatable by using the automated analytical syringe eVol® or connecting MEPS on-line with HPLC. MEPS also presents a cost per analysis minimal compared to conventional SPE because each MEPS sorbent can be re-used several times before being discarded while SPE cartridges are indicated for single use. MEPS also enables the reduction of the solvent volume consumption and the sample preparation time. On the other hand, a small volume of plasma (100 µL) is required in this case in comparison with many other methods published that use larger sample volumes (0.5-2 mL) (STOREY and HOLT, 1982; PLOMP *et al.,* 1983; GUPTA and CONNOLLY, 1984; MARCHISET *et al.,* 1984; SHIPE, 1984; HUTCHINGS, SPRAGG and ROUTLEDGE, 1986; SUSANTO, HUMFELD and REINAUER, 1986; LESNE and PELLEGRIN, 1987; DE SMET and MASSART, 1988; PACZKOWSKI *et al.,* 1989; ROCHAS *et al.,* 1989; VERBESSELT, TJANDRAMAGA and DE SCHEPPER 1991; GUMIENICZEK, MISZTAL and PRZYBOROWSKI, 1994; KOLLROSER and SCHOBER, 2002; RAJENDRAN *et al.,* 2006). In fact, our method presents a LOQ for AM and MDEA similar or even lower than other methods that employed a larger volume of plasma (PLOMP *et al.,* 1983; GUPTA and CONNOLLY, 1984; SHIPE, 1984; WEIR and UEDA, 1985; LESNE and PELLEGRIN, 1987; DE SMET and MASSART, 1988; GUMIENICZEK, MISZTAL and PRZYBOROWSKI, 1994; JUENKE *et al.,* 2004). However, there are also methods reported in literature presenting lower LOQs for AM and MDEA but making use of more expensive and sensitive detection systems, such as tandem mass spectrometry (KOLLROSER and SCHOBER, 2002; KUHN, GOTTING and KLEESIEK, 2010), or using higher volumes of sample (STOREY and HOLT, 1982; MARCHISET *et al.,* 1984; PACZKOWSKI *et al.,*

1989; ROCHAS *et al.,* 1989; VERBESSELT, TJANDRAMAGA and DE SCHEPPER*,* 1991; RAJENDRAN *et al.,* 2006; BOLDERMAN, HERMANS and MAESSEN*.,* 2009).

In addition, this bioanalytical method enables the rapid analysis of AM and MDEA in human plasma samples (less than 5 min) using the usual detection system and the simplest chromatography conditions found in clinical units. In this method the IS selected was TAM which is commercially available; in contrast, other analytical methods for determination of AM and MDEA used as IS a compound that is no longer available L8040 (a brominated analogue of AM) (STOREY and HOLT, 1982; PLOMP *et al.,* 1983; MARCHISET *et al.,* 1984; SHIPE, 1984; WEIR and UEDA, 1985; SUSANTO, HUMFELD and REINAUER*,* 1986; LESNE and PELLEGRIN, 1987; DE SMET and MASSART, 1988; PACZKOWSKI *et al.,* 1989; ROCHAS *et al.,* 1989; VERBESSELT, TJANDRAMAGA and DE SCHEPPER, 1991; JUENKE *et al.,* 2004).

This method was also successfully applied to real plasma samples of highly polymedicated patients receiving treatment with AM. From the analysis of the Table II.6 it is evident that in some patients the concentrations of AM are below the proposed therapeutic range (0.5-2.0 μg/mL) and in patient ID_3 the AM plasma concentration was found to be below the limit of quantification (BLQ; < 0.1 μg/mL). These findings may be explained by the short time of treatment with AM, since the patients have initiated AM therapy in the hospital few days (2-10 days) before sample collection. Indeed, as AM and MDEA have a huge apparent volume of distribution, it is likely to find BLQ levels at the end of the dosing interval during the first days of AM therapy. On the other hand, these results expose the urgent need for routine TDM of AM plasma/serum concentrations as a guide to individualize dosing regimens, even during the first days/weeks of treatment.

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CHAPTER III

A RAPID HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF AMIODARONE AND ITS MAJOR METABOLITE IN RAT PLASMA AND TISSUES: A USEFUL TOOL FOR PHARMACOKINETIC STUDIES

III. A RAPID HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF AMIODARONE AND ITS MAJOR METABOLITE IN RAT PLASMA AND TISSUES: A USEFUL TOOL FOR PHARMACOKINETIC STUDIES

III.1. ABSTRACT

A rapid and sensitive high-performance liquid chromatography (HPLC) method was developed and validated in rat plasma and tissue (heart, liver, kidney and lung) homogenates for the determination of amiodarone (AM) and its main metabolite (mono-*N*-desethylamiodarone, MDEA), using tamoxifen as internal standard. Chromatographic separation was achieved within less than 5 min on a LiChroCART Purospher® Star C_{18} column (55 mm × 4 mm, 3 µm). The mobile phase consisting of phosphate buffer (50 mM) with 0.1% formic acid (pH 3.1)/methanol/acetonitrile (45:5:50, v/v/v) was pumped isocratically at a flow-rate of 1.2 mL/min. The detection was conducted at 254 nm for all compounds. Calibration curves were linear ($r^2 \geq 0.995$) in the range of 0.1-15 µg/mL for AM and MDEA. The limits of quantification were established at 0.1 µg/mL for both analytes. The overall data of precision and accuracy were in accordance with international guidelines for bioanalytical method validation. AM and MDEA were extracted from rat matrices by a liquid-liquid extraction procedure and the mean recovery ranged from 59.9% to 97.6%. This novel HPLC method enables the fast and reliable determination of AM and MDEA from several rat matrices (plasma, liver, kidneys, lungs and heart) and was successfully applied in a preliminary pharmacokinetic study.

III.2. INTRODUCTION

Amiodarone (AM; Figure III.1), despite the well-known safety concerns ascribed to the drug itself or its major metabolite mono-*N*-desethylamiodarone (MDEA; Figure III.1), remains one of the most prescribed and efficacious antiarrhythmic agents (VASSALO and THROMAN, 2007; VAN HERENDAEL and DORIAN, 2010; FREEMANTLE *et al.,* 2011). Therefore, in spite of the recent clinical introduction of a new antiarrhythmic drug structurally related to AM – dronedarone – AM is expected to continue to be a valuable agent of the limited armamentarium of antiarrhythmic drugs. Indeed, some concerns about the long-term safety of dronedarone still persist (FOOD AND DRUG ADMINISTRATION, 2011).

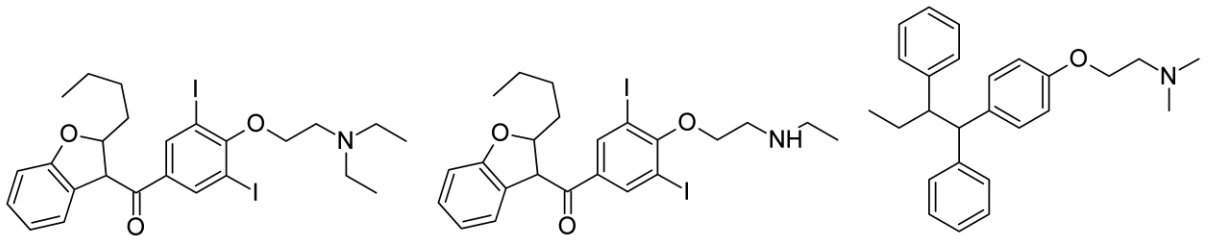

Figure III.1 – Chemical structures of amiodarone (AM), mono-*N*-desethylamiodarone (MDEA) and tamoxifen (TAM) used as internal standard (IS).

AM presents some unfavourable and very unusual properties from the pharmacokinetic viewpoint, which certainly determine the pharmacodynamic and toxicological profiles (FUKUCHI *et al.,* 2009; VAN HERENDAEL and DORIAN, 2010). In humans, AM has shown an erratic gastrointestinal absorption and, consequently, a variable oral bioavailability (20-80%) (VAN HERENDAEL and DORIAN, 2010). Moreover, AM has a huge body tissue distribution and a correspondingly long elimination half-life (WOLKOVE and BALTZAN, 2009). Additionally, AM has been recognised as a drug of narrow serum/plasma therapeutic range (0.5-2.0 μg/mL) (PÉREZ-RUIZ *et al.,* 2002; SHAYEGANPOUR, HAMDY and BROCKS, 2008) and has also been associated to important clinical drug interactions (ROUGHEAD *et al.,* 2010). AM has usually been considered the precipitant or interacting agent in the majority of pharmacokinetic-based interactions reported in literature, inducing a change (usually an increase) in serum/plasma levels of co-administered drugs [e.g. digoxin (TRUJILLO and NOLAN, 2000), warfarin (TRUJILLO and NOLAN, 2000), phenytoin (LESKO, 1989), theophylline (TRUJILLO and NOLAN, 2000) and simvastatin (MAROT *et al.,* 2011)]. Nevertheless, contrary to the

expectations, few studies have been published describing the interference of other compounds on the pharmacokinetics of AM; specifically, the metabolism of AM was dramatically inhibited by grapefruit juice (LIBERSA *et al.,* 2000), the systemic exposure to AM and MDEA was significantly reduced by the simultaneous administration with orlistat (ZHI *et al.,* 2003) and, more recently, the exposure of rats to β-naphthoflavone (a polycyclic aromatic hydrocarbon) was found to increase the formation of MDEA probably through cytochrome P450 (CYP) induction (ELSHERBINY, EL-KADI and BROCKS, 2010). Thus, it is urgent to investigate in more depth the potential for relevant drug interactions where AM is considered the target of interaction (object drug).

Because rat species share many of the pharmacokinetics properties of AM with humans, the rat has been regarded as one of the best models among the small laboratory animals for studying pharmaco-toxicological aspects concerning AM; although some metabolic differences exist, it has also been demonstrated that MDEA is the principal metabolite of AM in both species (PLOMP *et al.,* 1987; NAJJAR, 2001; ELSHERBINY and BROCKS, 2010; ELSHERBINY, EL-KADI and BROCKS, 2010). Hence, the availability of a simple, fast and inexpensive bioanalytical method to enable the reliable quantification of AM and its pharmacologically active metabolite MDEA in the matrices of rat plasma and tissues is essential to support many non-clinical assays.

Thus far, a few methods have been reported in literature for the simultaneous determination of AM and MDEA in rat plasma and tissues; in addition, the published methods require considerably long analysis times (AL-DHAWAILIE, 1995; JUN and BROCKS, 2001; SHAYEGANPOUR and BROCKS, 2003) and present other limitations such as complex and expensive sample pre-treatment procedures (MOOR, WYSS and BICKEL, 1988) or the use of costly analytical instrumentation [liquid chromatography-mass spectrometry (LC-MS)] which are not available in all laboratories (SHAYEGANPOUR, SOMAYAJI and BROCKS, 2007). Thus, a new liquid chromatography assay was herein developed in order to minimize a lot of limitations of the previous existing methods.

This work describes a high-performance liquid chromatography (HPLC) method which was developed and fully validated for the rapid and simultaneous quantification of AM and MDEA from samples of rat plasma and tissues (heart, liver, kidney and lung). Because the method requires small volumes of rat plasma, it is also a valuable tool for application in pharmacokinetic studies with AM in which multiple, serial blood sample collection is desired from individual rats.

III.3. EXPERIMENTAL

III.3.1 CHEMICALS AND REAGENTS

AM (lot no. 078K1246) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and MDEA (lot no. LB33020) was kindly supplied by Sanofi-Aventis (Paris, France). Tamoxifen (TAM; lot no. 035K1270), as citrate salt, was obtained from Sigma-Aldrich (St Louis, MO, USA) and it was used as internal standard (IS). The chemical structures of these compounds are shown in Figure III.1. Methanol (HPLC gradient grade) and acetonitrile (HPLC gradient grade) were purchased from Merck KGaA (Darmstadt, Germany) and VWR International (Leuven, Belgium), respectively. Ultra-pure water (HPLC grade, > 18 MΩ; home-made) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). All other reagents were of analytical grade: sodium phosphate monobasic purris. p.a. (Sigma-Aldrich GmbH; Seelze, Germany), sodium phosphate dibasic anhydrous (Sigma-Aldrich; St Louis, MO, USA), sodium dihydrogen phosphate dehydrate purum p.a. (Fluka Chemie; Buchs, Switzerland) and formic acid (98-100%) (BDH Chemicals; Poole, England).

III.3.2 BLANK RAT PLASMA AND TISSUES

Adult male Wistar rats, weighing 300-380 g, were obtained from local animal facilities (Faculty of Health Sciences of the University of Beira Interior). The rats were maintained under controlled environmental conditions (temperature 20±2 °C; relative humidity 55±5%; 12-h light/dark cycle). All animals were allowed free access to a standard diet (4RF21, Mucedola, Italy) and water *ad libitum* until the experimental procedures. Rats not subjected to other pharmacological treatments were previously anesthetized with an intraperitoneal (i.p.) injection of a mixture of ketamine (90 mg/kg)/xylazine (10 mg/kg), and then used as source of the blank plasma and tissue (heart, liver, kidney and lung) samples required in the validation studies. For that, blood samples were collected into heparinized tubes after decapitation of anesthetized rats. The plasma was separated by centrifugation at 4000 rpm for 10 min (4 °C) and stored at –20 °C until use. After exsanguination, liver, kidneys, heart and lungs were quickly removed, weighed and homogenized in distilled water (3 mL of water per gram of tissue); the tissue homogenates were also stored at –20 °C until use. All animal experimentation was conducted in accordance with the European Directive (2010/63/EU)

for the accommodation and care of laboratory animals and the experimental procedures were approved by the Portuguese Veterinary General Division.

III.3.3 STOCK SOLUTIONS, CALIBRATION STANDARDS AND QUALITY CONTROL SAMPLES

Stock solutions of AM, MDEA, and TAM (IS) at concentration of 1 mg/mL were individually prepared by dissolving appropriate amounts of each compound in methanol. The stock solutions of AM and MDEA were adequately diluted with methanol to give intermediate solutions at 100 µg/mL. Thereafter, stock and intermediate solutions were appropriately used to afford six combined spiking solutions at final concentrations of 0.75, 1.5, 3.75, 11.25, 37.5 and 112.5 µg/mL for AM and MDEA which were used to spike aliquots of blank rat plasma (150 µL), and also six combined spiking solutions at final concentrations of 2, 4, 10, 30, 100 and 300 µg/mL were used to spike aliquots of blank tissue (heart, liver, kidney and lung) homogenates (400 µL). These procedures allowed the preparation of calibration standards in all considered matrices at six different concentration levels 0.1, 0.2, 0.5, 1.5, 5 and 15 µg/mL for AM and MDEA. The stock solution of IS was daily diluted with water-methanol (60:40, v/v) in order to obtain a working solution at 50 µg/mL. All solutions were stored at 4 °C and protected from light for one month, except the IS working solution which was prepared on each day of analysis. Quality control (QC) samples were prepared independently in the same biological matrices [rat plasma and tissue (heart, liver, kidney and lung) homogenates].

III.3.4 APPARATUS AND CHROMATOGRAPHIC CONDITIONS

The chromatographic analysis was performed using a Waters HPLC system Milford, MA, USA) equipped with an in-line solvent degasser (AF), a quaternary pump with controller (model 600), a manual injector (Rheodyne 7725i) and a diode array detector (DAD–2996). All instrumental parts were automatically controlled by Empower software supplied from Waters Corporation (Milford, MA, USA).

The chromatographic separation of the two analytes (AM and MDEA) and IS was achieved in approximately 5 min, at room temperature, by isocratic elution with a mobile phase of phosphate buffer (50 mM) with 0.1% formic acid (pH 3.1)/methanol/acetonitrile

(45:5:50, v/v/v) pumped at a flow-rate of 1.2 mL/min, on a reversed-phase column LiChroCART Purospher Star (C_{18}; 55 mm x 4 mm; 3 µm particle size) purchased from Merck KGaA (Darmstadt, Germany). The mobile phase was filtered through a 0.45 µm filter and degassed ultrasonically for 15 min before use. The injection volume was 20 µL and the wavelength of 254 nm was selected for the detection of all compounds (AM, MDEA and IS).

III.3.5 SAMPLE PREPARATION AND EXTRACTION

The sample pre-treatment was previously optimized and the final conditions were as follows. Each aliquot (150 µL) of rat plasma samples was diluted with 150 µL of 0.1 M sodium phosphate buffer (pH 5) and spiked with 20 µL of the IS working solution (50 µg/mL). The mixture was added of 500 µL of *n*-hexane (used as liquid-liquid extraction solvent), vortex-mixed for 30 sec and centrifuged at 17000 rpm for 2 min at 4 °C. The upper organic layer was transferred to a clean glass tube and the sample was re-extracted twice more with *n*-hexane (500 µL each time) using the conditions previously described. The whole organic extract was evaporated to dryness under a nitrogen stream at 60 °C and the residue was reconstituted in 100 µL of methanol. Following this, an aliquot of the reconstituted extracts (20 µL) was injected into the HPLC system for analysis.

For the extraction from tissues, each aliquot (400 µL) of tissue (heart, liver, kidney and lung) homogenates was spiked with 20 µL of the IS working solution (50 µg/mL); then, the mixture was added of 400 µL of acetonitrile (used as protein precipitating agent), vortex-mixed for 1 min and centrifuged at 17000 rpm for 10 min at 4 °C in order to precipitate the protein content. The supernatant was transferred to a new propylene tube and 1 mL of *n*-hexane (used as liquid-liquid extraction solvent) was added. The mixture was vortex-mixed for 1 min and centrifuged at 17000 rpm for 5 min at 4 °C. The upper organic layer (*n*-hexane) was transferred to a clean glass tube and the sample was re-extracted twice more with *n*-hexane (0.8 mL each time) using the same conditions. The organic extract was evaporated to dryness, reconstituted and then injected into the HPLC system using the same procedures as mentioned above for rat plasma samples.

III.3.6 METHOD VALIDATION

The described method was validated according to the general recommendations published in the last few years concerning the bioanalytical method validation and acceptance criteria for validation parameters: selectivity, linearity, sensitivity, precision and accuracy, sample dilution, recovery and stability (SHAH *et al.,* 2000; GUIDANCE FOR INDUSTRY, 2001; EUROPEAN MEDICINES AGENCY, 2011).

Selectivity was evaluated by comparing the chromatograms generated after the analysis of blank samples (plasma and heart, liver, kidney and lung homogenates) obtained from six different rats with those achieved from the corresponding spiked samples in order to investigate the existence of potential chromatographic interferences from endogenous compounds (matrix effects) at the retention times of AM, MDEA and IS. Additionally, interferences from drugs (ketamine, xylazine and heparin) usually used in experimental protocols of pharmacokinetic studies were also tested by injecting standard solutions of these compounds.

The linearity of the analytical method was assessed in the range of 0.1-15 µg/mL for AM and MDEA. For that, calibration curves were prepared on five different days (n = 5) using spiked plasma and tissue (heart, liver, kidney and lung) homogenate calibration standards at six different concentration levels. Calibration curves were constructed by plotting the peak-area ratios (analyte/IS) as a function of the respective concentrations. The data were subjected to a weighted linear regression analysis using $1/x^2$ as the weighting factor for both analytes (AM and MDEA); this weighting factor was selected because it yielded the best fit of peak-area ratios *versus* concentration (ALMEIDA, CASTEL-BRANCO and FALCÃO, 2002). The limit of quantification (LOQ) for AM and MDEA, defined as the lowest concentration on the calibration curve that can be measured with acceptable intra and interday precision and accuracy, was assessed respectively by the percentage of coefficient of variation (% CV) not exceeding 20% and the percentage of deviation from nominal value (% *bias*) within ±20%. The LOQ was evaluated by analysing spiked plasma and tissue (heart, liver, kidney and lung) homogenate samples which were prepared in five replicates (n = 5). The limit of detection (LOD) of the analytes (AM and MDEA) in plasma and tissue (heart, liver, kidney and lung) homogenates was defined as the concentration that yields a signal-to-noise ratio of 3:1.

Intra and interday precision and accuracy were assessed by using QC samples analysed in replicate (n = 5) at three concentration levels (low, medium and high QC samples) representative of the calibration range; the concentrations tested were 0.3, 7.5 and 13.5 μg/mL for AM and MDEA. The acceptance criterion for intra and interday precision (expressed as % CV) was a CV not exceeding 15% (or 20% in the LOQ) and for accuracy (expressed as % *bias*) was a *bias* value within ±15% (or ±20% in the LOQ).

The dilution effect (1:4) was also investigated in plasma and tissue (heart, liver, kidney and lung) homogenates with appropriate QC samples at 25 μg/mL for AM and MDEA in order to ensure that samples exceeding the highest concentration (15 μg/mL) of the calibration range could be appropriately diluted with the respective blank matrix and, precisely and accurately quantified. The precision and accuracy of diluted samples were determined in both intra and interday assays (n = 5).

The recovery of the analytes (MDEA and AM) from plasma and tissue (heart, liver, kidney and lung) homogenate samples, submitted to the previously described pre-treatment procedures, was determined using QC samples at three concentration levels (low, medium and high QC samples) and analysed in replicate (n = 5). The recovery of the analytes was calculated by comparing the analyte peak-area of extracted QC samples against equivalent aqueous solutions. The recovery of the IS was also evaluated at the concentration used in the analysis of plasma and tissue homogenate samples.

The stability of AM and MDEA was assessed in all matrices (plasma and tissue homogenates), at low (QC_1) and high (QC_3) concentration levels, during 4 h at room temperature, 24 h at 4 °C, 30 days at –20 °C and –80 °C to simulate sample handling and storage time in the freezer before analysis (n = 5). Stability of the analytes (AM and MDEA) was also studied at 4 °C during 24 h in the processed samples (post-preparative stability in methanol) to reproduce the time that samples can be in the auto-sampler before analysis. Finally, the stability of AM and MDEA was also evaluated after three freeze-thaw cycles at –20 °C and –80 °C. Aliquots of spiked samples (QC_1 and QC_3) were stored at –20 °C and at –80 °C for 24 h, thawed unassisted at room temperature, and when completely thawed samples were refrozen for 24 h under the same conditions until three cycles were completed. Stability was assessed comparing the data of QC samples analysed before (reference samples) and after being exposed to the conditions for stability assessment (stability samples); a stability/reference samples ratio of 85-115% was accepted as stability criterion (n = 5).

III.3.7 METHOD APPLICATION AND PHARMACOKINETIC ANALYSIS

Following the oral administration of a single dose of AM to Wistar rats (300-380 g), the analytical method was applied to investigate the plasma pharmacokinetic profiles of AM and its major metabolite (MDEA), as well as AM and MDEA concentrations presented in the tissues (heart, liver, kidney and lung) at the end of the experiments (24 h post-dose). On the day before the pharmacokinetic study, a lateral tail vein of each rat (n = 5) was cannulated, under anaesthesia [ketamine (90 mg/kg)/xylazine (10 mg/kg); i.p. injection], by insertion of an Introcan® Certo IV indwelling cannula (22G; 0.9 x 2.5 mm) made of polyurethane (B. Braun Melsungen AG, Melsungen, Germany). The rats fully recovered from anaesthesia and were fasted overnight with free access to water. The day after, rats received 50 mg/kg of AM administered by oral gavage; the solution of AM (12.5 mg/mL) administered to the rats was extemporaneously prepared from a commercial injectable solution of AM (50 mg/mL), which was appropriately diluted with 5% dextrose solution. After the administration of AM, multiple serial blood samples (~0.3 mL) were collected into heparinized tubes at pre-specified post-dose time points (0.25, 0.5, 1, 2, 4, 6, 8 and 12 h) through the catheter previously introduced in lateral tail vein of rats. In addition, at 24 h post-dose, under anaesthesia, blood and tissue (heart, liver, kidney and lung) samples were also harvested after decapitation of rats. The blood and tissue samples were immediately processed as indicated in the *section III.3.2*. The plasma and tissue homogenates obtained were frozen at –20 °C until analysis. All these animal experiments were conducted in accordance with the European Directive (2010/63/EU) for the accommodation and care of laboratory animals and the experimental procedures were approved by the Portuguese Veterinary General Division.

The plasma concentration-time profiles obtained for AM and MDEA were submitted to a non-compartmental pharmacokinetic analysis using the WinNonlin® version 4.1 (Pharsight Co, Mountain View, CA, USA). The peak concentrations of AM and MDEA in plasma (C_{max}) and the time to reach C_{max} (t_{max}) were obtained directly from the experimental data. Other pharmacokinetic parameters were estimated from the plasma concentration data obtained at each time point (0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 h): area under the concentration-time curve (AUC) from time zero to the last sampling time at which concentrations were at or above the LOQ of the method (AUC_{0-t}), calculated by the linear trapezoidal rule; AUC from time zero to infinite ($AUC_{0-\infty}$), calculated from AUC_{0-t} + (C_{last}/k_{el}), where C_{last} is the last quantifiable concentration and k_{el} is the apparent

terminal elimination rate constant calculated by log-linear regression of the terminal segment of the concentration-time profile; apparent terminal elimination half-life ($t_{1/2}$) and mean residence time (MRT). The plasma concentrations of AM and MDEA lower than the LOQ of the assay were taken as zero for all calculations.

III.4. RESULTS

III.4.1 METHOD DEVELOPMENT

Once identified from the literature the scarcity of bioanalytical methods available for the analysis of AM and its main metabolite (MDEA) in rat plasma and tissues, as well as their major analytical drawbacks, the development of the method herein described was conducted aiming to overcome as much as possible the current analytical limitations. To achieve satisfactory chromatographic separation associated with short chromatographic running times, a short reversed-phase LiChroCART® Purospher Star-C_{18} column (55 mm x 4 mm; 3 µm particle size) was selected. In addition, the outstanding robustness and excellent pH-stability indicated by the manufacturer, enabling the development of methods across a wide pH range (1.5-10.5) were characteristics considered in the column selection. Then, to optimize the best chromatographic conditions to separate the analytes (MDEA and AM) in the column, individual and combined solutions of the compounds were directly injected into HPLC system and several mobile phases were tested using a standard flow rate (1 mL/min); at the beginning, the focus was the chromatographic resolution of AM and MDEA. First, the absence of buffer and salt in the mobile phase was considered aiming to reduce the pressure and prolong the column lifetime, avoiding the formation of precipitates due to the limited solubility of salts in organic modifiers. Thus, considering the information available in literature and aiming to minimize the run time while maintaining a good chromatographic resolution between adjacent peaks, different mobile phases were tested consisting of only water and acetonitrile as organic modifier. However, an adequate chromatographic resolution between MDEA and AM and peak symmetry was achieved only after changing the aqueous portion of the mobile phase to 50 mM phosphate buffer with a pH value near to 3 adjusted with formic acid. After that, the next step was the introduction of an IS (TAM); TAM was the compound selected to be used as IS because it is structurally related to analytes (AM and MDEA) and is commercially available; TAM showed a shorter retention time than the analytes by isocratic elution with a mobile phase of phosphate buffer (50 mM) with 0.1% formic acid (pH 3.1)/acetonitrile (50:50, v/v), but an overlap of TAM and MDEA chromatographic peaks was observed to an extent that prevented the individual peak integration. Trying to reach the complete chromatographic separation of the TAM and MDEA, a second organic modifier (methanol in a percentage of 5%) was included in the

composition of the mobile phase. A mobile phase composed by phosphate buffer (50 mM) with 0.1% formic acid (pH 3.1)/methanol/acetonitrile (45:5:50, v/v/v) allowed the complete resolution of the three compounds (TAM, MDEA and AM). The flow rate was then set at 1.2 mL/min (isocratic conditions) and all the compounds eluted in a short running time of approximately 5 min.

In addition to the optimization of the chromatographic conditions for the separation and detection of the IS and analytes (MDEA and AM) after use of standard solutions, the development of an analytical method to quantify drugs or metabolites in biological samples also requires the development of a suitable sample extraction procedure. In fact, sample pre-treatment is a vital step in bioanalysis, enabling the removal of proteins that can precipitate on the chromatographic column or eliminate potential matrix interferences. In this case, before selecting a liquid-liquid extraction (LLE) procedure to extract the analytes from rat plasma samples, other simpler and faster sample preparation approaches were tested, particularly the precipitation of proteins with methanol and acetonitrile; however, in these conditions, several peaks of endogenous substances appeared at the retention times of the compounds of interest (lacking selectivity). Afterward, to develop a simple and inexpensive analytical method, LLE procedures using low solvent volumes were considered instead of solid-phase extraction (SPE). Accordingly, several organic solvents (chloroform, dichloromethane, diethyl ether, ethyl acetate and *n*-hexane) were tested in order to optimize the selectivity and recovery. The organic solvent that allowed the best selectivity was *n*-hexane, and a good recovery was obtained by multiple (three) LLE steps using small volumes of solvent. Finally, the LLE procedure established for the preparation of rat plasma samples was selected, as previously described *(section III.3.5).* Because rat tissue (heart, liver, kidney and lung) samples are more complex than those of plasma, this makes the analysis of drugs or metabolites in such biological matrices more difficult and additional steps of sample preparation are usually required. Thus, to determine AM and MDEA concentrations in rat tissue samples, tissue homogenization was selected as the first step of tissue sample preparation. As for tissue homogenate samples the application of isolated procedures of protein precipitation or LLE did not provide enough chromatographic selectivity, the subsequent strategy consisted of a protein precipitation step followed by LLE procedures. Acceptable selectivity and recovery was achieved by protein precipitation with acetonitrile followed by LLE with *n*-hexane using the previously mentioned conditions *(section III.3.5).* Once available, an HPLC system coupled to a diode array detector (DAD) was used and the

maximum absorbance wavelengths of the analytes were investigated, presenting maximum absorbance at 240 nm for MDEA and AM, and at this wavelength the absorption of the IS was also near the maximum. However, to improve the selectivity of the analytical assay against the endogenous interferences of rat matrices, the wavelength selected was 254 nm instead of 240 nm.

III.4.2 METHOD VALIDATION

Selective extraction and chromatographic resolution of the compounds of interest (IS, MDEA and AM) from spiked rat plasma and tissue (heart, liver, kidney and lung) homogenate samples were successfully attained using the previously described sample preparation procedures and chromatographic conditions. Typical chromatograms obtained after analysis of the extracts of blank and spiked rat plasma and tissue homogenate samples are shown in Figure III.2. The chromatograms demonstrated that, in all blank rat matrices (plasma and heart, liver, kidney and lung tissue homogenates) no endogenous compounds interfered at the retention times of the IS and analytes (MDEA and AM). In addition, none of the tested drugs (ketamine, xylazine and heparin) frequently used in experimental protocols of pharmacokinetic studies were found to interfere at the retention times of the chromatographic peaks of IS, MDEA and AM.

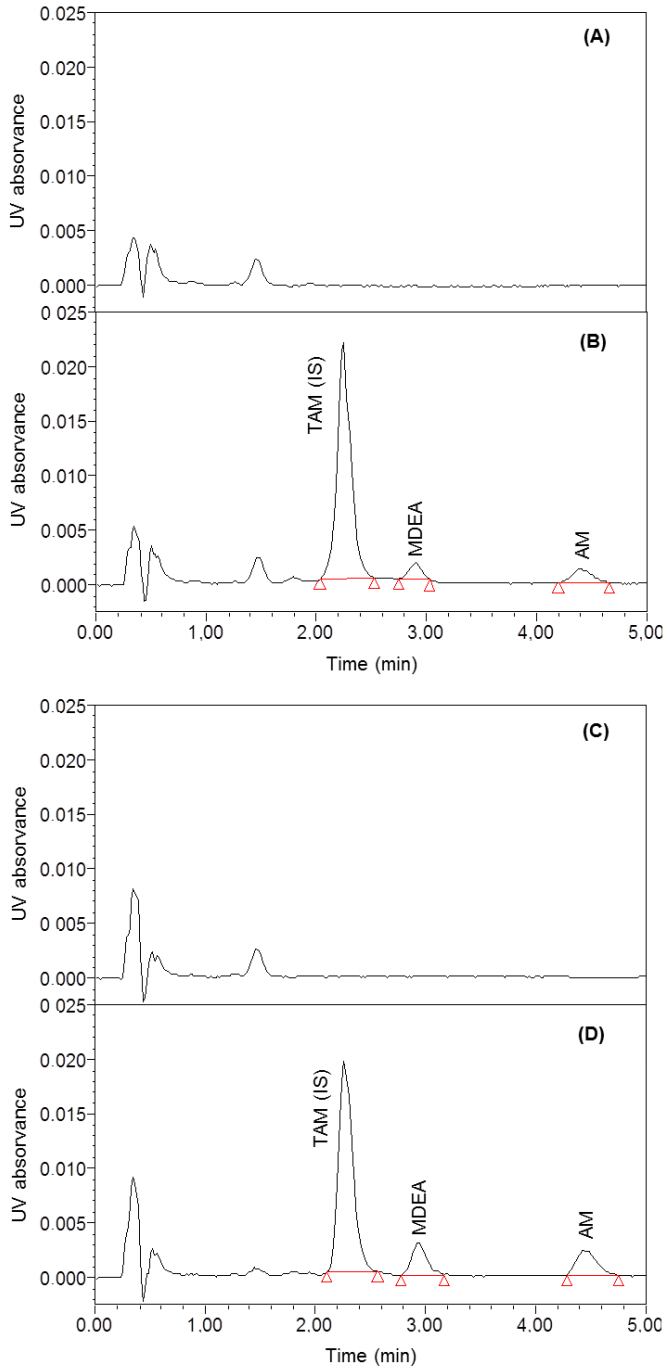

Figure III.2 – Typical chromatograms of extracted rat samples: blank plasma (A); plasma spiked with mono-*N*-desethylamiodarone (MDEA) and amiodarone (AM) at 0.5 µg/mL (B); blank heart tissue homogenate (C); heart tissue homogenate spiked with MDEA and AM at 0.5 µg/mL (D). As shown for heart tissue homogenate, similar chromatographic behaviour was obtained for corresponding samples of liver, kidney and lung tissue homogenates.

The linearity of the analytical method in the calibration range of 0.1-15 µg/mL was demonstrated for both compounds (AM and MDEA) in all rat matrices (plasma, and heart,

liver, kidney and lung tissue homogenates; $r^2 \geq 0.995$). The calibration curves were subjected to weighted linear regression analysis and the corresponding parameters are summarized in Table III.1. Under the experimental conditions described, the LOQ of the assay was set at 0.1 μg/mL for AM and MDEA in all rat matrices investigated with acceptable precision and accuracy as shown in Table III.2. The LOD of the analytes (AM and MDEA) was established at 0.02 μg/mL in plasma and at 0.01 μg/mL in tissue (heart, liver, kidney and lung) homogenates.

Table III.1 – Calibration curve parameters for mono-*N*-desethylamiodarone (MDEA) and amiodarone (AM) in rat matrices (plasma, and heart, liver, kidney and lung homogenates) (n = 5).[a]

Matrix/Analyte	Calibration standards (μg/mL)	Slope (b)	Intercept (a)	r^2
Plasma				
MDEA	0.1, 0.2, 0.5, 1.5, 5, 15	0.1394	–0.0019	0.998
AM	0.1, 0.2, 0.5, 1.5, 5, 15	0.1654	–0.0017	0.999
Heart homogenate				
MDEA	0.1, 0.2, 0.5, 1.5, 5, 15	0.3346	0.0028	0.999
AM	0.1, 0.2, 0.5, 1.5, 5, 15	0.3645	0.0016	0.999
Liver homogenate				
MDEA	0.1, 0.2, 0.5, 1.5, 5, 15	0.3542	0.0034	0.998
AM	0.1, 0.2, 0.5, 1.5, 5, 15	0.3598	–0.0057	0.995
Kidney homogenate				
MDEA	0.1, 0.2, 0.5, 1.5, 5, 15	0.3169	–0.0072	0.999
AM	0.1, 0.2, 0.5, 1.5, 5, 15	0.3443	–0.0059	0.998
Lung homogenate				
MDEA	0.1, 0.2, 0.5, 1.5, 5, 15	0.3823	–0.0049	0.998
AM	0.1, 0.2, 0.5, 1.5, 5, 15	0.3783	–0.0092	0.998

[a] Equation of the calibration curve: $y = bx + a$, where x is the analyte concentration, expressed in μg/mL, and y is the analyte to IS peak area ratio, expressed in arbitrary area units; r^2, regression coefficient.

The intra and interday precision and accuracy data of the assay using low, medium and high QC samples for plasma and tissue (heart, liver, kidney and lung) homogenates are shown in Table III.2. The overall intra and interday imprecision (% CV) not exceed 6.56% and the inaccuracy (% bias) was within ±7.94%. These data are in compliance with the international guidelines for the validation of bioanalytical methods. Therefore, the method is

precise and accurate for determination of AM and MDEA in rat plasma, and heart, liver, kidney and lung homogenate samples. The precision and accuracy was also demonstrated for the sample dilution (1:3) of plasma and tissue (heart, liver, kidney and lung) homogenates (Table III.2). These results show that a four-fold dilution with the corresponding blank rat matrices can be appropriately applied if the concentration of the analytes (AM and MDEA) in an assayed sample exceeds the highest concentration level of the calibration curve (15 µg/mL).

Table III.2 – Precision and accuracy for the determination of mono-*N*-desethylamiodarone (MDEA) and amiodarone (AM) in rat matrices (plasma, and heart, liver, kidney and lung homogenates) at the concentrations of the limit of quantification (*), at low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration range (n = 5), and following sample dilution (#) by a 4-fold factor.

Analyte	$C_{nominal}$ (µg/mL)	Precision (% CV)	Accuracy (% *bias*)	Precision (% CV)	Accuracy (% *bias*)	Precision (% CV)	Accuracy (% *bias*)	Precision (% CV)	Accuracy (% *bias*)	Precision (% CV)	Accuracy (% *bias*)
		Plasma	**Plasma**	**Heart homogenate**	**Heart homogenate**	**Liver homogenate**	**Liver homogenate**	**Kidney homogenate**	**Kidney homogenate**	**Lung homogenate**	**Lung homogenate**
Interday											
MDEA	0.1*	7.44	3.44	9.01	6.66	9.84	−9.53	8.82	0.30	8.79	4.65
	0.3	6.56	−6.17	1.62	6.94	6.46	−0.40	3.40	3.14	3.72	4.69
	7.5	2.23	3.51	1.60	2.44	3.27	5.18	1.88	1.16	3.49	1.15
	13.5	4.50	3.32	1.78	0.43	1.62	7.94	2.69	3.43	3.31	1.21
	25#	9.36	−2.72	3.14	−0.78	4.83	7.49	2.02	−1.39	5.54	1.53
AM	0.1*	9.77	−0.59	9.80	10.80	11.47	12.84	6.69	2.54	7.97	0.92
	0.3	3.38	−2.36	4.58	5.47	4.71	−4.38	4.22	4.39	2.65	4.41
	7.5	2.24	0.71	1.53	2.28	1.59	5.34	2.18	2.05	3.53	1.39
	13.5	3.07	−1.13	1.51	3.76	2.05	6.90	2.63	5.22	3.44	1.25
	25#	7.41	−3.69	2.67	−1.02	4.36	13.68	3.28	−1.96	3.32	1.42

Table III.2 – Precision and accuracy for the determination of mono-*N*-desethylamiodarone (MDEA) and amiodarone (AM) in rat matrices (plasma, and heart, liver, kidney and lung homogenates) at the concentrations of the limit of quantification (*), at low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration range (n = 5), and following sample dilution (#) by a 4-fold factor *(cont.)*.

Analyte	$C_{nominal}$ (µg/mL)	Precision (% CV)	Accuracy (% *bias*)	Precision (% CV)	Accuracy (% *bias*)	Precision (% CV)	Accuracy (% *bias*)	Precision (% CV)	Accuracy (% *bias*)	Precision (% CV)	Accuracy (% *bias*)
		Plasma	**Plasma**	**Heart homogenate**	**Heart homogenate**	**Liver homogenate**	**Liver homogenate**	**Kidney homogenate**	**Kidney homogenate**	**Lung homogenate**	**Lung homogenate**
Intraday											
MDEA	0.1*	8.98	7.92	3.92	2.17	5.49	−1.87	5.19	6.39	5.33	2.62
	0.3	6.23	−4.88	2.45	6.46	3.39	−4.57	1.88	6.36	1.93	5.51
	7.5	2.15	0.35	2.18	1.64	3.12	5.97	2.67	−0.69	2.84	−1.19
	13.5	2.35	−1.88	2.14	−1.36	1.18	5.32	2.36	1.62	3.45	−0.14
	25#	3.44	−0.53	2.65	−2.79	3.33	8.13	1.38	−3.10	2.28	−3.06
AM	0.1*	9.66	−0.51	9.06	−2.81	5.3	11.69	2.95	2.60	4.47	0.42
	0.3	5.13	−3.52	3.22	0.80	4.72	−3.29	2.08	3.59	4.65	7.31
	7.5	1.30	1.39	2.75	0.82	2.27	6.43	2.98	0.76	3.32	−1.09
	13.5	1.79	−2.92	2.16	1.19	1.56	5.65	2.48	3.48	3.44	−0.65
	25#	1.34	0.93	2.18	−4.19	3.58	14.76	1.37	−3.68	2.31	−1.04

$C_{nominal}$, nominal concentration.

The recovery values of AM and MDEA from rat matrices (plasma, and heart, liver, kidney and lung homogenates) are presented in Table III.3. The mean recoveries, taking AM and its major metabolite (MDEA) into account, ranged from 59.9% to 97.6%, and showed low CV values. The recovery of the IS from all rat matrices considered showed mean values between 72.6% and 91.7%, with CV values not exceeding 4.56%.

Table III.3 – Recovery (%) of mono-*N*-desethylamiodarone (MDEA) and amiodarone (AM) from rat matrices (plasma, and heart, liver, kidney and lung homogenates) at low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration ranges (n = 5).

Matrix/Analyte	$C_{nominal}$ (µg/mL)	Recovery (%)	
		Mean ± SD (n = 5)	CV (%)
Plasma			
MDEA	0.3	61.0 ± 4.23	6.93
	7.5	74.7 ± 0.70	0.94
	13.5	78.3 ± 2.20	2.81
AM	0.3	88.7 ± 3.90	4.39
	7.5	96.9 ± 2.11	2.18
	13.5	97.6 ± 2.52	2.58
Heart homogenate			
MDEA	0.3	59.9 ± 3.26	5.45
	7.5	71.0 ± 3.23	4.54
	13.5	77.0 ± 1.60	2.08
AM	0.3	76.7 ± 0.47	0.61
	7.5	85.3 ± 3.20	3.76
	13.5	90.4 ± 2.20	2.43
Liver homogenate			
MDEA	0.3	62.2 ± 3.42	5.50
	7.5	61.5 ± 1.37	2.22
	13.5	64.6 ± 3.93	6.09
AM	0.3	63.4 ± 6.31	9.95
	7.5	75.6 ± 1.67	2.22
	13.5	79.3 ± 4.61	5.82

Table III.3 – Recovery (%) of mono-*N*-desethylamiodarone (MDEA) and amiodarone (AM) from rat matrices (plasma, and heart, liver, kidney and lung homogenates) at low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration ranges (n = 5) *(cont.).*

Matrix/Analyte	$C_{nominal}$ (µg/mL)	Recovery (%)	
		Mean ± SD (n = 5)	CV (%)
Kidney homogenate			
MDEA	0.3	61.6 ± 3.62	5.88
	7.5	72.0 ± 1.68	2.33
	13.5	70.0 ± 1.89	2.70
AM	0.3	73.7 ± 2.53	3.44
	7.5	84.4 ± 1.81	2.15
	13.5	83.0 ± 2.67	3.22
Lung homogenate			
MDEA	0.3	79.8 ± 3.40	4.26
	7.5	83.6 ± 0.78	0.93
	13.5	83.8 ± 1.83	2.18
AM	0.3	81.8 ± 4.85	5.93
	7.5	87.2 ± 0.94	1.08
	13.5	86.6 ± 1.65	1.91

$C_{nominal}$, nominal concentration ; SD, standard deviation.

From the stability data obtained in rat plasma and tissue (heart, liver, kidney and lung) homogenates no significant loss was observed for AM and MDEA when spiked QC samples (QC_1 and QC_3) were stored at room temperature for 4 h, at 4 °C for 24 h, and at –20 °C and –80 °C for 30 days. The analytes (AM and MDEA) also demonstrated to be stable after three freeze-thaw cycles at –20 °C and –80 °C; in addition, no significant degradation occurred in processed samples (methanol extract) stored at 4 °C for 24 h. Stability data are summarized in Table III.4.

Table III.4 – Stability (values in percentage) of mono-*N*-desethylamiodarone (MDEA) and amiodarone (AM) in unprocessed rat matrices (plasma, and heart, liver, kidney and lung homogenates) at room temperature for 4 h, at 4 °C for 24 h, after three freeze-thaw cycles (–20 °C and –80 °C) and at –20 °C and –80 °C for 30 days; and in processed rat matrices (plasma, and heart, liver, kidney and lung homogenates) left at 4 °C for 24 h (n = 5).

Analyte	MDEA		AM	
$C_{nominal}$ (μg/mL)	0.3	13.5	0.3	13.5
Unprocessed Plasma				
Room temperature (4 h)	100.7	97.0	104.6	95.4
4 °C (24 h)	101.8	94.6	102.6	95.2
Freeze-thaw (3 cycles; –20 °C)	104.7	94.7	103.6	96.5
Freeze-thaw (3 cycles; –80 °C)	101.5	109.3	104.3	111.8
–20 °C (30 days)	92.5	92.6	98.8	91.1
–80 °C (30 days)	89.5	93.3	94.0	97.0
Processed Plasma				
4 °C (24 h)	110.9	104.7	111.0	102.6
Unprocessed heart homogenate				
Room temperature (4 h)	104.5	103.5	105.1	103.4
4 °C (24 h)	102.6	97.4	100.5	97.1
Freeze-thaw (3 cycles; –20 °C)	100.9	89.3	106.2	101.5
Freeze-thaw (3 cycles; –80 °C)	101.3	96.2	101.3	95.2
–20 °C (30 days)	95.3	91.2	111.5	89.7
–80 °C (30 days)	92.4	91.1	107.1	89.0
Processed heart homogenate				
4 °C (24 h)	104.3	95.8	104.1	96.3
Unprocessed liver homogenate				
Room temperature (4 h)	99.2	106.6	100.6	104.9
4 °C (24 h)	99.5	98.9	103.1	100.3
Freeze-thaw (3 cycles; –20 °C)	110.6	110.9	94.5	111.6
Freeze-thaw (3 cycles; –80 °C)	113.1	107.9	98.6	89.5
–20 °C (30 days)	103.2	101.9	93.0	102.3
–80 °C (30 days)	108.3	97.6	98.3	98.9
Processed liver homogenate				
4 °C (24 h)	107.4	108.2	103.0	107.6

Table III.4 – Stability (values in percentage) of mono-*N*-desethylamiodarone (MDEA) and amiodarone (AM) in unprocessed rat matrices (plasma, and heart, liver, kidney and lung homogenates) at room temperature for 4 h, at 4 °C for 24 h, after three freeze-thaw cycles (–20 °C and –80 °C) and at –20 °C and –80 °C for 30 days; and in processed rat matrices (plasma, and heart, liver, kidney and lung homogenates) left at 4 °C for 24 h (n = 5) *(cont.).*

Analyte	MDEA		AM	
$C_{nominal}$ (μg/mL)	0.3	13.5	0.3	13.5
Unprocessed kidney homogenate				
Room temperature (4 h)	96.6	96.1	98.6	97.0
4 °C (24 h)	96.7	93.8	93.7	94.1
Freeze-thaw (3 cycles; –20 °C)	95.3	91.7	86.2	93.2
Freeze-thaw (3 cycles; –80 °C)	96.2	90.7	86.3	93.9
–20 °C (30 days)	94.3	92.5	86.4	90.5
–80 °C (30 days)	99.9	92.8	89.6	91.8
Processed kidney homogenate				
4 °C (24 h)	105.2	99.0	90.4	96.5
Unprocessed lung homogenate				
Room temperature (4 h)	95.9	96.5	100.6	97.9
4 °C (24 h)	97.8	94.5	102.8	94.0
Freeze-thaw (3 cycles; –20 °C)	110.6	99.7	110.2	96.7
Freeze-thaw (3 cycles; –80 °C)	110.0	101.2	107.9	99.9
–20 °C (30 days)	86.3	94.5	90.2	85.4
–80 °C (30 days)	93.3	92.2	95.6	89.1
Processed lung homogenate				
4 °C (24 h)	110.8	101.8	108.7	101.3

$C_{nominal}$, nominal concentration.

III.4.3 METHOD APPLICATION AND PHARMACOKINETIC STUDY

The HPLC method was successfully applied to the analysis of MDEA and AM in serial plasma samples taken over a period of time of 24 h from Wistar rats (n = 5) administered with a single oral dose of AM (50 mg/kg). The blood sample collection performed at 24 h post-dose involved a terminal procedure, and then, heart, liver, kidney and lung tissues were

excised after exsanguinations, allowing also the application of the method to the determination of AM and MDEA concentrations in rat tissue samples.

The plasma concentration-time profiles obtained for AM and MDEA from each rat are depicted in Figure III.3. In Figure III.4 are also shown the corresponding mean plasma concentration-time profiles (n = 5) and the most significant pharmacokinetic parameters of AM and MDEA estimated by non-compartmental analysis are summarized in Table III.5. The concentrations of AM and MDEA measured in tissue (heart, liver, kidney and lung) and plasma samples at 24 h post-dose are shown in Table III.6.

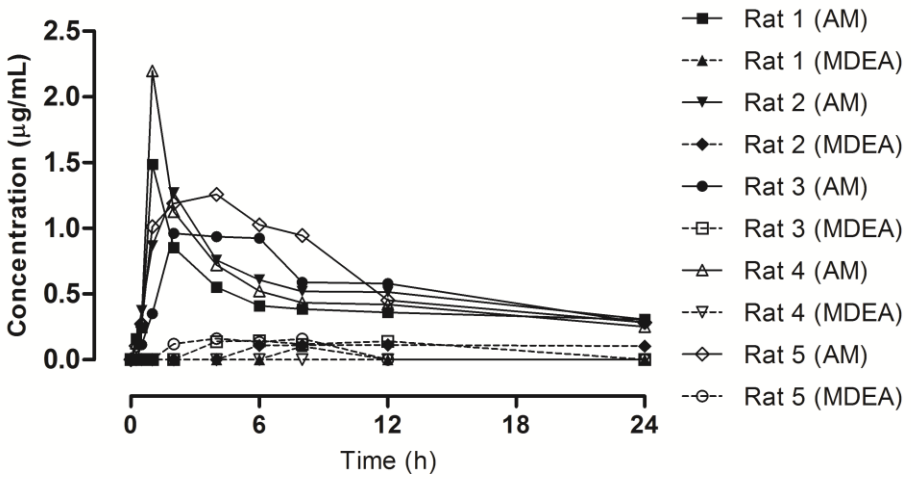

Figure III.3 – Individual plasma concentration-time profiles of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA), over a period of 24 h, obtained from rats treated with a single dose of 50 mg/kg AM administered by oral gavage (n = 5).

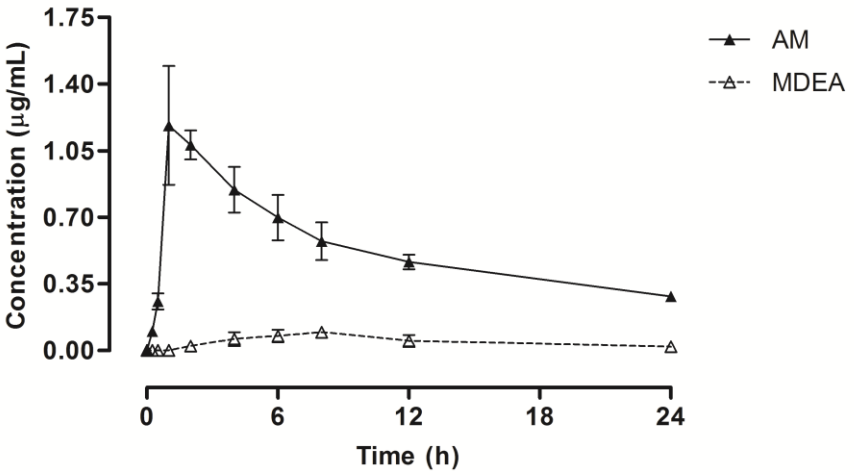

Figure III.4 – Mean plasma concentration-time profiles of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA), over a period of 24 h, obtained from rats treated with a single dose of 50 mg/kg AM administered by oral gavage. Symbols represent the mean values ± standard error of the mean (SEM) of five determinations per time point (n = 5).

Table III.5 – Pharmacokinetic parameters estimated by non-compartmental analysis of the mean plasma concentration-time profiles of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) in rats after a single oral dose of 50 mg/kg of AM.

Pharmacokinetic parameters[a]	AM	MDEA
Plasma		
t_{max} (h)	1.0	8.0
C_{max} (µg/mL)	1.183	0.097
AUC_{0-t} (µg.h/mL)	12.868	1.129
$AUC_{0-\infty}$ (µg.h/mL)	19.484	1.342
$t_{1/2el}$ (h)	16.1	7.4
MRT (h)	22.1	14.1

[a]C_{max} and t_{max} are experimental values; AUC_{0-t}, $AUC_{0-\infty}$, $t_{1/2el}$ and MRT values were calculated by non-compartmental analysis from mean concentrations at each time point (n = 5).

Table III.6 – Plasma and tissue (heart, liver, kidney and lung) concentrations of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) at 24 h post-dose in rats after a single oral dose of 50 mg/kg of AM (*n* = 5).

Matrix/Concentration (24 h)	AM	MDEA
Plasma (μg/mL)		
Rat 1	0.305	0.000
Rat 2	0.306	0.102
Rat 3	0.280	0.000
Rat 4	0.250	0.000
Rat 5	0.283	0.000
Mean	**0.285**	**0.020**
SD	0.0229	0.0456
Heart tissue (μg/g)		
Rat 1	4.342	1.870
Rat 2	4.795	2.134
Rat 3	4.105	1.541
Rat 4	4.071	1.598
Rat 5	4.273	2.144
Mean	**4.317**	**1.857**
SD	0.2903	0.2854
Liver tissue (μg/g)		
Rat 1	6.812	2.235
Rat 2	7.492	2.582
Rat 3	5.952	1.880
Rat 4	6.565	1.852
Rat 5	6.536	2.383
Mean	**6.671**	**2.186**
SD	0.5568	0.3176

Table III.6 – Plasma and tissue (heart, liver, kidney and lung) concentrations of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) at 24 h post-dose in rats after a single oral dose of 50 mg/kg of AM (*n* = 5) *(cont).*

Matrix/Concentration (24 h)	AM	MDEA
Kidney tissue (µg/g)		
Rat 1	8.562	4.912
Rat 2	8.990	5.107
Rat 3	7.436	3.876
Rat 4	6.541	3.413
Rat 5	8.221	5.449
Mean	**7.950**	**4.551**
SD	0.9721	0.8654
Lung tissue (µg/g)		
Rat 1	32.686	22.618
Rat 2	33.544	24.839
Rat 3	26.987	16.980
Rat 4	33.598	21.306
Rat 5	31.591	24.410
Mean	**31.681**	**22.031**
SD	2.7477	3.1596

SD, Standard deviation.

III.5. DISCUSSION

The HPLC method herein described presents important bioanalytical improvements over the few assays previously published for the quantification of AM and MDEA in rat plasma and tissue samples (MOOR, WYSS and BICKEL, 1988; AL-DHAWAILIE, 1995; JUN and BROCKS, 2001; SHAYEGANPOUR, SOMAYAJI and BROCKS, 2007) and the method was successfully applied to a preliminary pharmacokinetic study. The analytical method was fully validated in rat plasma and heart, liver, kidney and lung tissue homogenates, enabling the fast chromatographic analysis of AM and MDEA in such biological samples (in approximately 5 min) using simple instrumentation and uncomplicated chromatographic conditions (isocratic elution and detection in UV spectral region); these aspects suggest that the method is appropriate for implementation in almost all laboratories, and fast bioanalytical assays are essential whenever a large number of samples have to be analysed. At this point, the full validation performed in all rat tissue matrices considered (heart, liver, kidney and lung) may not be necessary. Indeed, taking into account the general recommendations published in last few years concerning bioanalytical method validation, a partial validation could be acceptable for different matrices within a species (SHAH *et al.,* 2000; GUIDANCE FOR INDUSTRY, 2001; EUROPEAN MEDICINES AGENCY, 2011). However, for the reliable application of this method to pharmacokinetic assays, the validation parameters selectivity, intraday precision and accuracy, and stability should be evaluated in any case in all intended biological matrices. Therefore, since the bioanalytical method was under development and implementation for the first time, a full validation was always considered to ensure greater confidence in the data.

In this method, the plasma sample preparation only involves LLE, and for tissue sample preparation, an additional step of protein precipitation is employed. These procedures are significantly less expensive than those of the method developed by MOOR, WYSS and BICKEL (1988) which combines protein precipitation and SPE for both plasma and tissue samples. Despite the method described by MOOR, WYSS and BICKEL (1988) appears to be more sensitive for plasma (0.03 µg/mL as LOQ) than the method herein reported (0.1 µg/mL as LOQ), it is necessary to take into account that the plasma sample volume used for analysis is higher in the first (500 µL *versus* 150 µL). This aspect is determinant when serial blood sample collection in survival, non-terminal pharmacokinetic studies is required; indeed, 500 µL plasma sample volume is too large, compromising an experimental design involving

serial blood sample collection. AL-DHAWAILIE (1995) reported a method in which an appropriate volume of plasma to perform pharmacokinetic studies (100 µL) was used, but it was validated exclusively for the determination of AM, disregarding the main pharmacologically and toxicologically active metabolite (MDEA), and also presented as disadvantage a time-consuming chromatographic analysis (~14 min). JUN and BROCKS (2001) also developed and validated a method requiring small volumes of rat plasma (100 µL), but exclusively for the determination of AM. This method was improved some years later by SHAYEGANPOUR and BROCKS (2003) introducing also the metabolite (MDEA); however, the primary drawback was not overcome, because long chromatographic analysis times remained (~14 min). More recently, SHAYEGANPOUR, SOMAYAJI and BROCKS (2007) developed another method for AM and MDEA in rat plasma and tissues using an LC-MS assay, which required more expensive instrumentation that may not be available in all labs. In addition, in the method developed by SHAYEGANPOUR, SOMAYAJI and BROCKS (2007) the volume of *n*-hexane required for LLE was approximately double that used in this method.

As demonstrated in this work, the present method is a useful tool to support rat pharmacokinetic-based studies with AM, making it possible to characterize AM and MDEA plasma pharmacokinetic profiles in each rat. As expected, AM has shown an erratic and variable absorption from rat gastrointestinal tract (Figure III.3). In agreement with SHAYEGANPOUR, SOMAYAJI and BROCKS (2007), this preliminary pharmacokinetic study also showed that systemic exposure to MDEA (major metabolite) is much lower than that to AM, even following oral administration of the parent compound. Bearing in mind the plasma and tissue (heart, liver, kidney and lung) concentration data measured at 24 h post-dose (Table III.6), it was evident that higher concentrations of AM and MDEA were found in tissues than in plasma, supporting their greater plasma-tissue distribution. Additionally, these findings also support the extensive accumulation of AM and MDEA in rat lung tissue, which may explain why pulmonary toxicity is one of the most serious toxic effects associated with AM treatment. Indeed, the highest concentrations of AM and MDEA were observed in the rat lung and, at this level, the huge MDEA concentrations determined in lung tissue (more than 1000-fold) comparatively to those found in plasma. The results of this pharmacokinetic study corroborate the conclusions of recent non-clinical studies that have also linked the metabolite MDEA to pulmonary cytotoxicity induced by AM (ELSHERBINY, EL-KADI and BROCKS, 2010; MULDER *et al.,* 2011).

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CHAPTER IV

HERB-DRUG INTERACTION OF FUCUS VESICULOSUS EXTRACT AND AMIODARONE IN RATS: A POTENTIAL RISK FOR REDUCED BIOAVAILABILITY OF AMIODARONE IN CLINICAL PRACTICE

IV. HERB-DRUG INTERACTION OF *FUCUS VESICULOSUS* EXTRACT AND AMIODARONE IN RATS: A POTENTIAL RISK FOR REDUCED BIOAVAILABILITY OF AMIODARONE IN CLINICAL PRACTICE

IV.1. ABSTRACT

Fucus vesiculosus is a seaweed claimed to be useful for obesity management. Therefore, considering the relationship between obesity and cardiovascular diseases, this work aimed to assess the potential for an herb-drug interaction among a standardized *F. vesiculosus* extract (GMP certificate) and amiodarone (AM; a narrow therapeutic index drug) in rats. In a first pharmacokinetic study, rats were simultaneously co-administered with a single-dose of *F. vesiculosus* (575 mg/kg, p.o.) and AM (50 mg/kg, p.o.); in a second study, rats were pre-treated during 14 days with *F. vesiculosus* (575 mg/kg/day, p.o.) and received AM (50 mg/kg, p.o.) on the 15th day. Rats of the control groups received the corresponding volume of vehicle. After analysis of the pharmacokinetic data it deserves to be highlighted the significant decrease in the peak plasma concentration of AM (55.4%) as well as the reduction of systemic exposure to the parent drug (~30%) following the simultaneous co-administration of *F. vesiculosus* extract and AM. For the first time was reported an herb-drug interaction between *F. vesiculosus* and AM which determined a considerable decrease on AM bioavailability in rats. Therefore, the therapeutic efficacy of AM may be compromised by the concurrent administration of herbal slimming medicines/dietary supplements containing *F. vesiculosus.*

IV.2. INTRODUCTION

Amiodarone [2-*n*-butyl-3-(3,5-diiodo-4-diethylaminoethoxy-benzoyl)-benzofuran; AM] (Figure IV.1) is one of the most commonly prescribed antiarrhythmic drugs (VASSALLO and TROHMAN, 2007; VAN HERENDAEL and DORIAN, 2010; FREEMANTLE *et al.,* 2011). Despite the well-known safety concerns ascribed to AM and its major metabolite [mono-*N*-desethylamiodarone (MDEA); (Figure IV.1)], mainly pulmonary and hepatic toxicity as well as thyroid dysfunctions, AM has been considered the drug with best efficacy for the prophylaxis and treatment of a wide range of heart rhythm disorders (VAN HERENDAEL and DORIAN, 2010; FREEMANTLE *et al.,* 2011). In particular, AM is indicated for the treatment of severe rhythm disorders when other treatments are ineffective or have not been tolerated (VAN HERENDAEL and DORIAN, 2010).

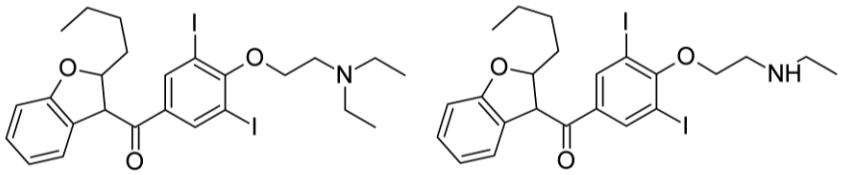

Figure IV.1 – Chemical structures of amiodarone (AM) and its major metabolite mono-*N*-desethylamiodarone (MDEA).

However, AM has some unfavourable and very unusual properties from the pharmacokinetic viewpoint, which certainly determine its pharmacodynamic and toxicological profiles (FUKUCHI *et al.,* 2009; VAN HERENDAEL and DORIAN, 2010). In humans, AM has shown an erratic gastrointestinal absorption and, consequently, a variable oral bioavailability (SHAYEGANPOUR, HAMDY and BROCKS, 2008; WOLKOVE and BALTZAN, 2009); this antiarrhythmic agent has also a huge body tissue distribution (OHYAMA *et al.,* 2000; VAN HERENDAEL and DORIAN, 2010) and a long elimination half-life (SHAYEGANPOUR, HAMDY and BROCKS, 2008). Moreover, AM has been recognised as a drug of narrow serum/plasma therapeutic range (0.5-2.0 μg/mL) (LESNE and PELLEGRIN, 1987; HUY *et al.,* 1991; MANFREDI *et al.,* 1995; IERVASI *et al.,* 1997; PÉREZ-RUIZ *et al.,* 2002; SHAYEGANPOUR, HAMDY and BROCKS, 2008) and has also been associated to important clinical drug interactions (EDWIN, JENNINGS and KALUS, 2010; KARIMI *et al.,* 2010; ROUGHEAD *et al.,* 2010). Nonetheless, AM has usually been considered the precipitant or interacting agent in the majority of pharmacokinetic-based drug interactions reported in literature, inducing a change (generally an increase) in

serum/plasma levels of co-administered drugs [e.g. digoxin (LESKO, 1989; TRUJILLO and NOLAN, 2000), warfarin (LESKO, 1989; TRUJILLO and NOLAN, 2000; YAMREUDEEWONG *et al.,* 2003), phenytoin (LESKO, 1989), theophylline (TRUJILLO and NOLAN, 2000) and simvastatin (BECQUEMONT *et al.,* 2007; MAROT *et al.,* 2011)]. Effectively, only few studies have been published describing the interference of other compounds on the pharmacokinetics of AM. Specifically, the metabolism of AM was dramatically inhibited by grapefruit juice (LIBERSA *et al.,* 2000); the systemic exposure to AM and MDEA was significantly reduced by the simultaneous administration of orlistat (ZHI *et al.,* 2003); and, more recently, the exposure of rats to β-naphthoflavone (a polycyclic aromatic hydrocarbon) was found to increase the formation of MDEA probably through cytochrome P450 (CYP) induction (ELSHERBINY, EL-KADI and BROCKS, 2010).

Clinical, pathophysiologic and epidemiological studies have clearly indicated that overweight and obesity are two of the major independent risk factors for coronary heart disease, atrial fibrillation and heart failure (SCAGLIONE *et al.,* 2004; ZALESIN *et al.,* 2011). Thus, given the increasing prevalence of obesity, reaching pandemic proportions in developed countries, it is expected a significant increase in the incidence of cardiovascular diseases (BODARY, IGLAY and EITZMAN, 2007), as well as in the clinical use of drugs such as AM (SINGH, 2008). On the other hand, aiming to improve their cardiovascular status, the patients are also increasingly seeking complementary medicines for weight reduction and/or weight management, including herbal dietary supplements. Hence, over the last years, the consumption of herbal weight loss supplements has grown at an alarming rate never seen before (EGRAS *et al.,* 2011).

Fucus vesiculosus, traditionally known as bladderwrack, is a medicinal plant that is claimed to be useful for the treatment of obesity, mainly due to its high levels in iodine, whose action is thought to be related to the stimulation of the thyroid gland and the subsequent effect on metabolic rate. Although iodine is considered the most prominent active ingredient of *F. vesiculosus*, the mucilage (dietary fibre), phytosterols and tetraterpenes are also important constituents responsible for its use in obesity management (MORO and BASILE, 2000; DÍAZ-RUBIO, PÉREZ-JIMÉNEZ and SAURA-CALIXTO, 2009). In fact, based on *F. vesiculosus* powder, the major components are non-digestible polysaccharides composed in a large extent by both soluble (10.52%) and insoluble (48.63%) fractions of dietary fibre (DÍAZ-RUBIO PÉREZ-JIMÉNEZ and SAURA-CALIXTO, 2009). In addition, besides proteins, minerals, vitamins and fatty acids, *F. vesiculosus* also contains polyphenols

(ZARAGOZÁ *et al.,* 2008; DÍAZ-RUBIO, PÉREZ-JIMÉNEZ and SAURA-CALIXTO, 2009) and some of them were recently found to inhibit drug-metabolizing enzymes like CYP1A (PARYS *et al.,* 2010).

For all the reasons referred to above, and bearing in mind the high potential for co-administration of *F. vesiculosus* medicinal/dietary preparations and AM, particularly in overweight or obese patients with cardiovascular diseases, it is urgent to generate data in order to assess the interference of *F. vesiculosus* on the pharmacokinetics of AM (a narrow therapeutic index drug). Furthermore, to the best of our knowledge, no study was previously conducted to evaluate the potential of interaction between *F. vesiculosus* and AM. Therefore, the present work was designed to investigate whether a commercial standardized *F. vesiculosus* extract may influence the pharmacokinetics of AM in rats, after their simultaneous oral co-administration, and following a 14-day *F. vesiculosus* pre-treatment period.

IV.3. MATERIALS AND METHODS

IV.3.1 DRUGS AND MATERIALS

Bladderwrack *(F. vesiculosus L.)* 0.10% dry aqueous extract (certificate of analysis number 201003344; provided as *Supplementary data*) obtained from thallus was purchased from EPO Istituto Farmochimico Fitoterapico s.r.l. (Milano, Italy). Carboxymethylcellulose sodium salt for preparation of extract suspension was obtained from Sigma (St. Louis, MO, USA). A commercial formulation (ampoules) of AM hydrochloride 50 mg/mL solution for intravenous injection was used for oral administration to rats after appropriate dilution with 5% glucose intravenous solution for infusion (B.Braun Medical, Portugal). Other compounds used were sodium chloride 0.9% solution for injection (Labesfal, Portugal); heparin sodium 5000 U.I./mL for injection (B.Braun Medical, Portugal); ketamine for injection (Imalgene 1000) and xylazine for injection (Vetaxilaze 20). Introcan® Certo IV indwelling cannula (22G; 0.9 × 2.5 mm) made of polyurethane from B. Braun (Melsungen AG, Melsungen, Germany).

IV.3.2 ANIMALS

Adult male Wistar rats (342 ± 28 g) of approximately 10 weeks old were obtained from local animal facilities (Faculty of Health Sciences of the University of Beira Interior, Covilhã, Portugal). The rats were maintained under controlled environmental conditions (temperature 20±2 °C; relative humidity 55±5%; 12-h light/dark cycle). The animals were allowed free access to a standard rodent diet (4RF21, Mucedola, Italy) during almost all experimental procedures and tap water was available *ad libitum*. At night on the day before dosing with AM, a lateral tail vein of each rat was cannulated under anaesthesia [ketamine (90 mg/kg)/xylazine (10 mg/kg); i.p. injection] by insertion of an Introcan® Certo IV indwelling cannula (22G; 0.9 × 2.5 mm) used for serial blood sampling. The rats fully recovered from anaesthesia overnight and were fasted for 12-14 h before AM administration and maintained with free access to water; an additional fasting period (4 h post-dose) was considered to avoid the effect of food on the oral bioavailability of AM. Oral treatments of the rats with *F. vesiculosus* and AM were performed by gavage. Blood sampling was conducted in conscious and freely moving rats, which were appropriately restrained only at the moment of blood collection, except for the last blood sampling that was taken by a terminal procedure

(decapitation and exsanguination under anaesthesia). All the animal experiments were conducted in accordance with the European Directive (2010/63/EU) for animal experiments and the experimental procedures were reviewed and approved by the Portuguese Veterinary General Division.

IV.3.3 EXPERIMENTAL DESIGN AND PHARMACOKINETIC STUDIES

To investigate the effects of *F. vesiculosus* on the kinetics of AM, two separate and independent pharmacokinetic studies were designed: (1) a single oral co-administration study of *F. vesiculosus* extract and AM; and (2) a 14-day repeated oral pre-treatment study with *F. vesiculosus* extract followed by a single oral administration of AM on the 15th day. The dose of *F. vesiculosus* was selected according to the study of SKIBOLA (2004) and taking into account the Food and Drug Administration (FDA) Guidance for Industry on conversion of animal doses to human equivalent doses based on body surface area (GUIDANCE FOR INDUSTRY, 2005); additionally, a 10-fold potentiating interaction factor was considered. On the other hand, the oral dose of AM (50 mg/kg) was established because it has provided plasma concentrations of drug in rats within the plasma therapeutic range (SHAYEGANPOUR, JUN and BROCKS, 2005). In each day of the experiments *F. vesiculosus* extract was suspended in 0.5% carboxymethylcellulose aqueous solution affording a suspension of herbal extract at 57.5 mg/mL. AM commercial injectable solution (50 mg/mL) was also appropriately diluted with 5% glucose solution to extemporaneously prepare an AM solution at 12.5 mg/mL. At this point, it should be noted that an AM injectable solution was used as starting drug material for oral administration, rather than AM tablets, to avoid the possible interference of tablet excipients on the bioavailability of AM in rats, thus ensuring better reproducibility, practicability and minimizing the presence of confounding factors that hindered the interpretation of the results. Appropriate volumes of *F. vesiculosus* extract suspension (10 mL/kg of body weight) and of AM solution (4 mL/kg of body weight) were orally administered to rats by gavage.

In the first pharmacokinetic study, twelve Wistar rats were randomly divided into two groups (experimental and control groups). Rats of the experimental group (n = 6) were concomitantly treated with a single-dose of *F. vesiculosus* extract (575 mg/kg, p.o.) and a single-dose of AM (50 mg/kg, p.o.); the extract suspension was administered right before AM. Rats of the control group (n = 6) received, instead of the *F. vesiculosus* extract

suspension, the corresponding volume of 0.5% carboxymethylcellulose aqueous solution (vehicle of the extract).

In the second pharmacokinetic study, twelve Wistar rats were also randomly divided into two groups. Rats assigned to the experimental group (n = 6) were orally pre-treated with *F. vesiculosus* extract (575 mg/kg, p.o.) once daily for 14 consecutive days (sub-chronic pre-treatment). Rats allocated to the control group (n = 6) were administered with an equivalent volume of vehicle for the same period of time. During the pre-treatment period, the rats were kept in 12-h light/dark cycle animal room with controlled temperature and humidity, as indicated above *(section IV.3.2);* free access to a standard rodent diet and tap water was allowed. On 15th day, rats of both groups (experimental and control) were gavaged with a single dose of AM (50 mg/kg, p.o.).

In both pharmacokinetic studies, the treatments with *F. vesiculosus* extract (or vehicle) and/or AM were always carried out on the morning between 9:00 am and 11:45 am. At night on the day before AM administration, the rats were anaesthetized for cannulation of a lateral tail vein and were fasted overnight as described above *(section IV.3.2).* On the day after, multiple serial blood samples (approximately 0.3 mL) were collected through the cannula into heparinized tubes before dosing and at 0.25, 0.5, 1, 2, 4, 6, 8 and 12 h following AM administration; then, at 24 h post-dose, blood and tissues (heart, liver, kidney and lung) were also harvested after decapitation of the rats. The blood samples were centrifuged at 4000 rpm for 10 min (4 °C) to separate the plasma which was stored at –20 °C until analysis. After exsanguination, liver, kidneys, heart and lungs were excised and stored at –20 °C; the organs were weighed and homogenized in distilled water (3 mL of water per gram of tissue) before analysis of tissue homogenates samples.

IV.3.4 DRUG ANALYSIS

Plasma and tissue concentrations of AM and its main metabolite (MDEA) were determined by using a liquid-liquid extraction (LLE) procedure followed by high-performance liquid chromatography-diode array detection (HPLC-DAD) assay previously developed and validated (RODRIGUES *et al.,* 2013). Briefly, an aliquot of each plasma sample (150 µL) was diluted with 150 µL of 0.1 M sodium phosphate buffer (pH 5) and spiked with 20 µL of the IS working solution (50 µg/mL). The mixture was added of 500 µL of *n*-hexane (used as LLE solvent), vortex-mixed for 30 sec and centrifuged at 17000 rpm for 2 min at 4 °C. The upper

organic layer was transferred to a clean glass tube and the sample was re-extracted two more times with *n*-hexane (500 µL each time) using the same experimental conditions. Then, the whole organic extract was evaporated to dryness under a nitrogen stream at 60 °C and the residue was reconstituted in 100 µL of methanol. Following this, an aliquot of the reconstituted extracts (20 µL) was injected into the HPLC system for analysis.

For the extraction from tissues, each aliquot (400 µL) of tissue (heart, liver, kidney and lung) homogenates was spiked with 20 µL of the IS working solution (50 µg/mL); then, the mixture was added of 400 µL of acetonitrile (used as protein precipitating agent), vortex-mixed for 1 min and centrifuged at 17000 rpm for 10 min at 4 °C in order to precipitate the protein content. The supernatant was transferred to a new propylene tube and 1 mL of *n*-hexane (used as LLE solvent) was added. The mixture was vortex-mixed for 1 min and centrifuged at 17000 rpm for 5 min at 4 °C. The upper organic layer (*n*-hexane) was transferred to a clean glass tube and the sample was re-extracted two more times with *n*-hexane (0.8 mL each time) using the same conditions. The organic extract was evaporated to dryness, reconstituted, and then injected into the HPLC system using the same procedures as mentioned above for rat plasma samples. The limit of quantification (LOQ) was established at 0.100 µg/mL for AM and MDEA in plasma and in tissue homogenates.

IV.3.5 PHARMACOKINETIC ANALYSIS

The plasma concentration *versus* time data for AM and MDEA obtained from each individual rat were submitted to a non-compartmental pharmacokinetic analysis using the WinNonlin® version 4.1 (Pharsight Co, Mountain View, CA, USA). The peak concentrations of AM and MDEA in plasma (C_{max}) and the time to reach C_{max} (t_{max}) were obtained directly from the experimental data. Other pharmacokinetic parameters estimated from the individual plasma concentration-time profiles were: area under the concentration-time curve (AUC) from time zero to the last sampling time at which concentrations were at or above the LOQ of the method (AUC_{0-t}), calculated by the linear trapezoidal rule; AUC from time zero to infinite ($AUC_{0-\infty}$), calculated from AUC_{0-t} + (C_{last}/k_{el}), where C_{last} is the last quantifiable concentration and k_{el} is the apparent terminal elimination rate constant calculated by log-linear regression of the terminal segment of the concentration-time profile; apparent terminal elimination half-life ($t_{1/2el}$) and mean residence time (MRT). The concentrations lower than the LOQ of the assay were taken as zero for all calculations.

IV.3.6 EFFECT OF THE SUB-CHRONIC *FUCUS VESICULOSUS* TREATMENT ON BODY WEIGHT

For the sub-chronic treatment study (a 14-day *F. vesiculosus* treatment period), the body weight of the rats receiving *F. vesiculosus* extract (575 mg/kg/day, p.o.; experimental group) or vehicle (control group) was adequately registered on the first day and on the last day (14^{th}) of these treatments in order to examine the effect of *F. vesiculosus* extract on body weight changes.

IV.3.7 STATISTICAL ANALYSIS

Data were reported as the mean ± standard error of the mean (SEM). Comparisons between two groups were usually performed using unpaired two-tailed Student's *t*-test; for body weight change comparisons within the same group the paired Student's *t*-test was employed. A difference was considered to be statistically significant for a *p*-value lower than 0.05 ($p < 0.05$).

IV.4. RESULTS

IV.4.1 EFFECTS OF THE SIMULTANEOUS CO-ADMINISTRATION OF *FUCUS VESICULOSUS* ON AMIODARONE PHARMACOKINETICS

The mean plasma concentration-time profiles (n = 6) of AM and its main metabolite (MDEA) obtained after intragastric co-administration of rats with a single-dose of *F. vesiculosus* extract (575 mg/kg, p.o.) or vehicle (control group) and a single-dose of AM (50 mg/kg, p.o.) are shown in Figure IV.2.

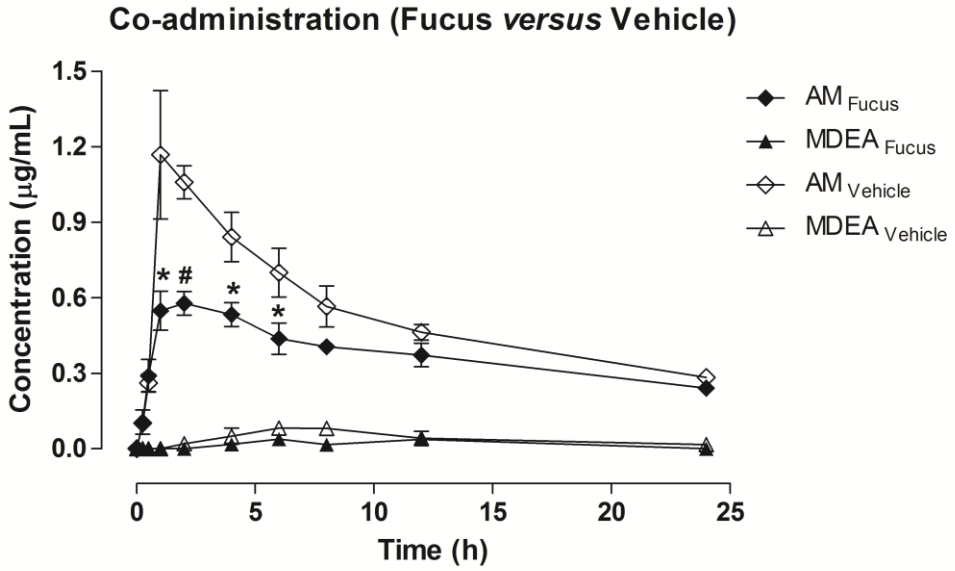

Figure IV.2 – Mean plasma concentration-time profiles of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) obtained, over a period of 24 h, from rats simultaneously treated in single-dose with *Fucus vesiculosus* extract (575 mg/kg, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and AM (50 mg/kg, p.o.) by oral gavage. Symbols represent the mean values ± standard error of the mean (SEM) of six determinations per time point (n = 6). $^{*}p < 0.05$ and $^{\#}p < 0.005$ compared to control (vehicle).

AM plasma concentrations were comparable in both groups at the initial absorption phase (up to 0.5 h) and at the elimination phase (8-24 h). Conversely, AM plasma concentrations in the group treated with *F. vesiculosus* were significantly lower than those in the control group over the 1-6 h post-dose time period (at least, $p < 0.05$). In the case of MDEA, the plasma concentrations were similar in both groups, with values near or below the LOQ (0.100 µg/mL) of the method. The main plasma pharmacokinetic parameters

estimated for AM and MDEA after non-compartmental analysis of their concentration-time profiles are summarized in Table IV.1.

Table IV.1 – Pharmacokinetic parameters estimated by non-compartmental analysis of the plasma concentration-time profiles of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA, major metabolite of AM) obtained in rats after the simultaneous co-administration in single-dose of *Fucus vesiculosus* extract (575 mg/kg, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), with AM (50 mg/kg, p.o.) by oral gavage (n = 6, unless otherwise noted).

Parameter	AM_{Fucus}		$AM_{Vehicle}$	
	AM	MDEA	AM	MDEA
t_{max} (h)	3.00 ± 1.10	8.00 ± 2.00[a]	1.83 ± 0.48	7.20 ± 1.36[b]
C_{max} (µg/mL)	0.615 ± 0.060*	0.114 ± 0.004[a]	1.378 ± 0.179	0.125 ± 0.012[b]
AUC_{0-t} (µg.h/mL)	8.995 ± 0.725*	ND	12.774 ± 0.688	ND
$AUC_{0-\infty}$ (µg.h/mL)	15.675 ± 1.722[#]	ND	21.431 ± 2.077	ND
k_{el} (1/h)	0.0426 ± 0.0070	ND	0.0433 ± 0.0082	ND
$t_{1/2el}$ (h)	18.29 ± 2.47	ND	20.73 ± 5.74	ND
MRT (h)	27.24 ± 3.45	ND	28.64 ± 7.74	ND

ND, not determined.
[a]n = 3; [b]n = 5.
*p < 0.005, significantly different from the control group.
[#]p = 0.059, *versus* control group.

With co-administration of *F. vesiculosus* the mean C_{max} of AM was significantly lower than that obtained in the control (vehicle) group (p < 0.005), while the mean time to reach t_{max} was attained later in the experimental group (3.00 ± 1.10 h) comparatively to the control group (1.83 ± 0.48 h). Statistically significant differences were also found for the AUC_{0-t} pharmacokinetic parameter (p < 0.005) calculated from the plasma concentration-time data obtained for AM in both groups; in contrast, these differences were not so evident for the $AUC_{0-\infty}$ parameter (p = 0.059) (Table IV.1). Taking into consideration the information derived from Figure IV.3, it is clear that following the simultaneous co-administration of *F. vesiculosus* and AM a remarkable decrease (55.4%) in the C_{max} of drug was observed, as well as a reduction of 29.6% in the extent of systemic drug exposure (as assessed by AUC_{0-t}).

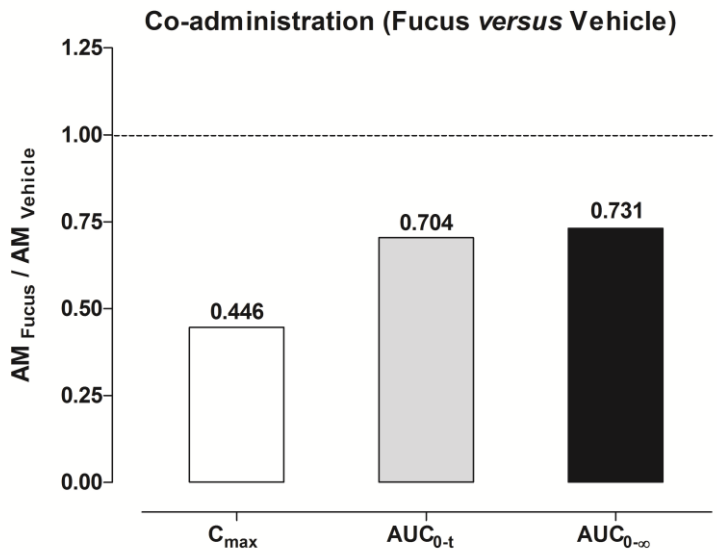

Figure IV.3 – Ratios for the main plasma pharmacokinetic parameters (C_{max}, AUC_{0-t} and $AUC_{0-\infty}$) estimated for amiodarone (AM) in rats simultaneously treated in single-dose with *Fucus vesiculosus* extract (575 mg/kg, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and AM (50 mg/kg, p.o.) by oral gavage.

As suggested by the visual inspection of the elimination phase of plasma pharmacokinetic profiles of AM (Figure IV.2), the mean values estimated for the elimination pharmacokinetic parameters (k_{el}, $t_{1/2el}$ and MRT) are comparable between experimental (*F. vesiculosus*) and control (vehicle) groups. Considering the scarcity of quantifiable plasma concentrations for MDEA obtained in both groups, only the C_{max} and t_{max} pharmacokinetic parameters are presented in Table IV.1.

On the other hand, to investigate some aspects related to the biodistribution of AM and MDEA in rats, the animals were killed at 24 h after dosing either in the presence or absence of the co-administration of *F. vesiculosus* and several tissues were excised and analysed. The mean concentrations of AM and MDEA determined in heart, lung, liver and kidney tissues, and also their plasma concentrations at the same time point (24 h), are shown in Figure IV.4.

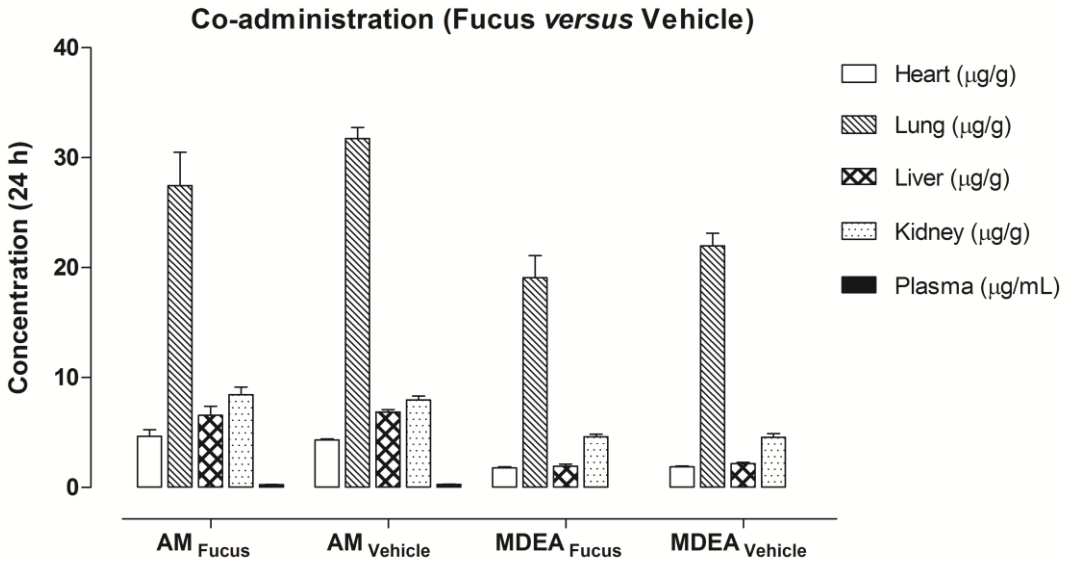

Figure IV.4 – Mean plasma and tissue (heart, lung, liver and kidney) concentrations of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) obtained, at 24 h post-dose, from rats simultaneously treated in single-dose with *Fucus vesiculosus* extract (575 mg/kg, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and AM (50 mg/kg, p.o.) by oral gavage. Data are expressed as the mean values ± standard error of the mean (SEM) of six determinations (n = 6).

As indicated in Figure IV.4, the tissue concentrations of AM and MDEA were markedly higher than those determined in plasma, and were absolutely noteworthy the levels found for both compounds (AM and MDEA) in the lung tissue. However, no significant differences were found in the concentrations of AM and MDEA in tissues (heart, liver, kidney and lung) collected from experimental (*F. vesiculosus*) and control (vehicle) groups (p > 0.05) at 24 h post-dose.

IV.4.2 EFFECTS OF THE SUB-CHRONIC PRE-TREATMENT WITH *FUCUS VESICULOSUS* ON AMIODARONE PHARMACOKINETICS

Rats were administered for 14 days with *F. vesiculosus* extract (575 mg/kg, p.o.) or vehicle (control group) in order to investigate a possible interference of the *F. vesiculosus* sub-chronic treatment on the pharmacokinetics of AM. The animals were given 50 mg/kg AM (p.o.) one day after the last treatment with *F. vesiculosus* or vehicle, and the mean

plasma concentration-time profiles (n = 6) of AM and its main metabolite (MDEA) are depicted in Figure IV.5.

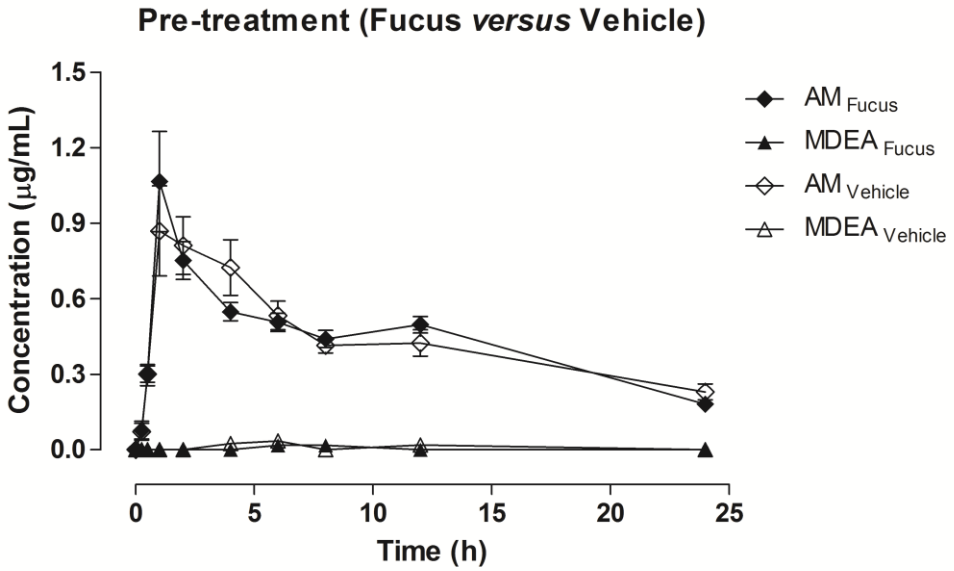

Figure IV.5 – Mean plasma concentration-time profiles of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) obtained, over a period of 24 h, from rats submitted to a 14-day pre-treatment period with *Fucus vesiculosus* extract (575 mg/kg/day, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and treated on the 15th day with a single-dose of AM (50 mg/kg, p.o.) by oral gavage (n = 6). Symbols represent the mean values ± standard error of the mean (SEM) of six determinations per time point (n = 6).

The corresponding pharmacokinetic parameters, calculated by using non-compartmental analysis, are listed in Table IV.2.

Table IV.2 – Pharmacokinetic parameters estimated by non-compartmental analysis of the plasma concentration-time profiles of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA, major metabolite of AM) obtained in rats submitted to a 14-day pre-treatment period with *Fucus vesiculosus* extract (575 mg/kg/day, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and treated on the 15th day with a single-dose of AM (50 mg/kg, p.o.) by oral gavage (n = 6, unless otherwise noted).

Parameter	AM_{Fucus}		$AM_{Vehicle}$	
	AM	MDEA	AM	MDEA
t_{max} (h)	1.00 ± 0.00	7.00 ± 1.00[a]	2.17 ± 0.60	7.33 ± 2.40[b]
C_{max} (µg/mL)	1.066 ± 0.199	0.108 ± 0.002[a]	0.952 ± 0.157	0.119 ± 0.014[b]
AUC_{0-t} (µg.h/mL)	10.555 ± 0.674	ND	10.532 ± 0.889	ND
$AUC_{0-\infty}$ (µg.h/mL)	13.786 ± 0.720	ND	15.325 ± 0.949	ND
k_{el} (1/h)	0.0594 ± 0.0051	ND	0.0533 ± 0.0082	ND
$t_{1/2el}$ (h)	12.15 ± 1.12	ND	14.36 ± 1.85	ND
MRT (h)	17.10 ± 1.45	ND	20.97 ± 2.42	ND

ND, not determined.
[a]n = 2; [b]n = 3.

Overall, it was observed a close overlap between the plasma pharmacokinetic profiles and no significant differences (p > 0.05) in the pharmacokinetic parameters were detected for AM and its main metabolite (MDEA) among the two groups (*F. vesiculosus versus* vehicle pre-treatment). Once again, the plasma concentrations of MDEA were near or below the LOQ (0.100 µg/mL) of the method in both groups of rats. Regarding the data shown in Figure IV.6, it is clear that the magnitude (as assessed by C_{max}) and the extent (as assessed by AUC) of systemic exposure to AM is similar among experimental and control groups (AM_{Fucus} *versus* $AM_{Vehicle}$ ratios near to unity).

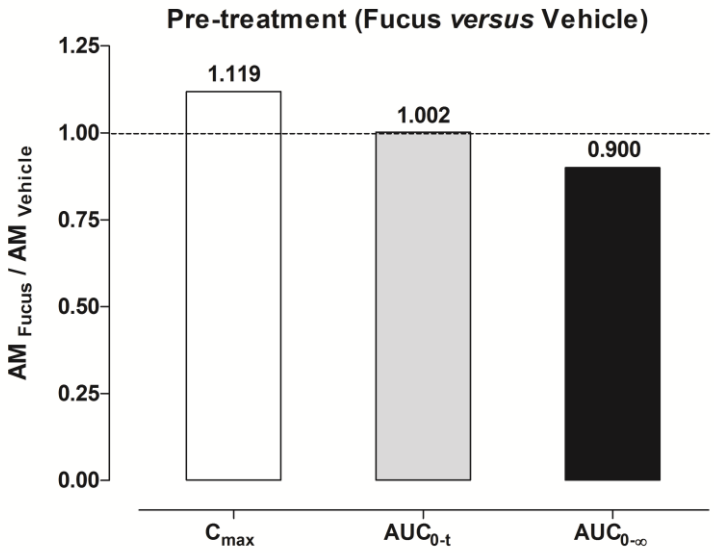

Figure IV.6 – Ratios for the main plasma pharmacokinetic parameters (C_{max}, AUC_{0-t} and $AUC_{0-\infty}$) estimated for amiodarone (AM) in rats submitted to a 14-day pre-treatment period with *Fucus vesiculosus* extract (575 mg/kg/day, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and treated on the 15th day with a single-dose of AM (50 mg/kg, p.o.) by oral gavage.

To examine the influence of a 14-day pre-treatment period with *F. vesiculosus* or vehicle (control group) on the distribution and metabolism of AM in rats, the concentrations of AM and its major metabolite (MDEA) were also determined in various tissues (additionally to plasma) at 24 h post-dose and the resultant mean concentrations are represented in Figure IV.7.

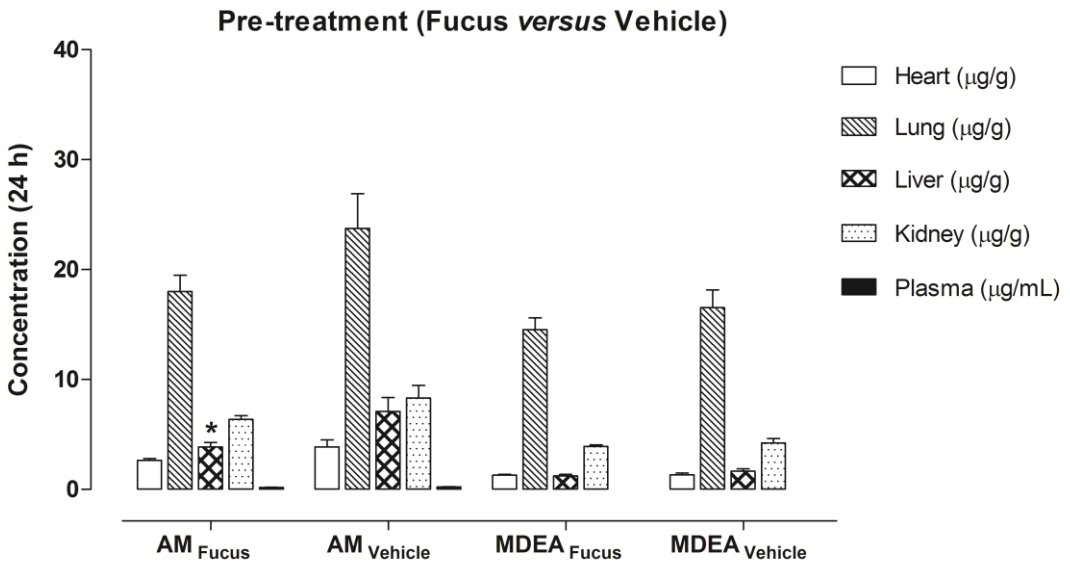

Figure IV.7 – Mean plasma and tissue (heart, lung, liver and kidney) concentrations of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) obtained, at 24 h post-dose, from rats submitted to a 14-day pre-treatment period with *Fucus vesiculosus* extract (575 mg/kg/day, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and treated on the 15th day with a single-dose of AM (50 mg/kg, p.o.) by oral gavage. Data are expressed as the mean values ± standard error of the mean (SEM) of six determinations (n = 6). *p < 0.05 compared to control (vehicle).

As pointed out in Figure IV.7, the concentrations of both compounds (AM and MDEA) in tissues were distinctly greater than those measured in plasma, and the concentration levels found in lung tissue were exceptionally high in experimental (*F. vesiculosus*) and control (vehicle) groups. However, taking into account all the comparisons performed, only significant differences were detected between both groups for AM concentrations in liver tissue, being such concentrations lower in liver tissue of rats treated with *F. vesiculosus* extract (p < 0.05).

IV.4.3. EFFECT OF THE SUB-CHRONIC *FUCUS VESICULOSUS* TREATMENT ON BODY WEIGHT

The resulting changes in body weight of the rats submitted to a 14-day treatment period with *F. vesiculosus* extract (575 mg/kg/day, p.o) or vehicle are demonstrated in Figure IV.8.

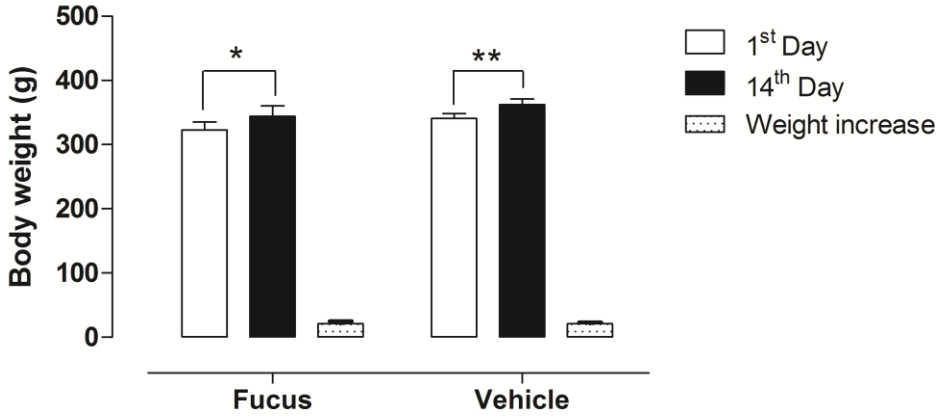

Figure IV.8 – Effects on the body weight of the rats induced by the sub-chronic treatment (14-day period) with *Fucus vesiculosus* extract (575 mg/kg/day, p.o.) and vehicle (0.5% carboxymethylcellulose aqueous solution) by oral gavage. *$p < 0.01$ and **$p < 0.001$, 1st day *versus* 14th day.

From the analysis of the data it is clear that the increase in body weight of rats treated with *F. vesiculosus* ($p < 0.005$) or treated with vehicle ($p < 0.0005$) was statistically significant between the 1st and 14th day. On the other hand, the increase in body weight of the rats of both groups (*F. vesiculosus versus* vehicle) was comparable.

IV.5. DISCUSSION

Complementary and alternative therapies such as herbal or natural products are finding increasing use around the world despite the paucity of scientific evidence about their safety and efficacy. Accordingly, unexpected and significant herb-drug interactions may occur and put individuals at risk, particularly those people who use multiple medicines for co-morbid conditions. These interactions may lead to therapeutic failure and/or toxic effects, especially for drugs characterized by a narrow therapeutic index (e.g. AM) (TACHJIAN, MARIA and JAHANGIR, 2010). Undoubtedly, the information now available on herb-drug interactions is scarce and, in many cases, inappropriate. Indeed, the information published about herb-drug interactions has derived mainly from *in vitro* studies; however, as suggested by some authors, numerous herb-drug interactions reported in literature are irrelevant and misleading due to the use of inappropriately high concentrations of the extracts or their constituents lacking, therefore, *in vivo* relevance when their bioavailability is considered (COTT, 2008; MARKOWITZ, VON MOLTKE and DONOVAN, 2008). Since the *in vitro* data on herb-drug interactions cannot usually be directly extrapolated to the *in vivo* conditions, it is important to conduct well-designed *in vivo* non-clinical studies with enough potency for human extrapolation.

Although safety issues are always important in the areas of pharmaceutical and food research, some safety data are already available in the literature for *F. vesiculosus* extracts. Indeed, acute toxicity studies performed in rats by ZARAGOZÁ *et al.* (2008) using two *F. vesiculosus* extracts indicated LD50 values of 1000-2000 (or >2000) mg/kg after oral dosing; on the other hand, the overall results obtained from a 4 week (sub-chronic) toxicity study conducted in rats with two oral doses of 200 mg/kg/day (low dose) and 750 mg/kg/day (high dose) indicated that no relevant signs of toxicity occurred even at the daily dose of 750 mg/kg (ZARAGOZÁ *et al.,* 2008). Moreover, LEITE-SILVA *et al.* (2007) demonstrated the absence of *F. vesiculosus* extract-mediated genotoxicity in cultured human lymphocytes and also evidenced its antigenotoxic activity against doxorubicin-induced DNA damage. PARYS *et al.* (2010) also reported potential chemopreventive activity of three fucophlorethols from *F. vesiculosus*. Thus, no safety issues were expected and none was observed at the dose of *F. vesiculosus* extract selected for the present study (575 mg/kg/day).

The current work was planned to investigate *in vivo* the potential of interaction between *F. vesiculosus* extract and AM, using adult male Wistar rat as a whole-animal model.

The use of female rats was also hypothesized during the experimental design of these studies; however, only male Wistar rats were included because it has been reported that the pharmacokinetics of AM is not gender-dependent and also to avoid the potential interference of menstrual cycle hormones (possible confounding factors) (SANOFI-SYNTHELABO PFIZER CANADA INC., 2010). Bearing in mind that drug-drug or herb-drug interactions mainly occur at the level of absorption and/or metabolic (inhibition or induction) pathways, the pharmacokinetic studies reported herein were designed to examine the interference of *F. vesiculosus* extract on the gastrointestinal absorption (simultaneous co-administration study) and metabolism of AM (14-day *F. vesiculosus* pre-treatment study).

Our results clearly evidenced a significant decrease (55.4%) in the peak plasma concentration (C_{max}) of drug following the simultaneous co-administration of the *F. vesiculosus* extract and AM, as well as a reduction of approximately 30% in the extent of systemic drug exposure (as assessed by $AUC_{0\text{-}24h}$). On the other hand, no important effects were detected either on the magnitude or extent of systemic exposure to AM after the administration of the drug to pre-treated rats one day after the last treatment with *F. vesiculosus* extract or vehicle. Hence, taking these findings together, it is apparent that *F. vesiculosus* extract or its components interact with AM in the gastrointestinal tract, reducing significantly the bioavailability of the drug; actually, this interaction was identified as relevant only after the simultaneous co-administration in single-dose of *F. vesiculosus* extract and AM. Moreover, the similarity in plasma pharmacokinetic profiles of AM obtained from rats pre-treated for 14 days with *F. vesiculosus* extract or vehicle excludes the impact of *F. vesiculosus*-induced metabolism on the bioavailability and also supports the significance of the interaction with AM at the level of the gastrointestinal tract. ZHI *et al.* (2003) also observed in healthy volunteers a significant reduction of the absorption of AM induced by orlistat; this drug, a lipase inhibitor, significantly reduced the systemic exposure to AM by approximately 25% and a decrease of similar magnitude (~25%) was detected in the generation of the metabolite MDEA (the major metabolite of AM). According to ZHI *et al.* (2003) the absorption of highly lipophilic drugs such as AM may depend on the presence of a lipid phase in the gastrointestinal environment, which may be affected by the pharmacological action of orlistat. In fact, there are strong evidences supporting the effects of food upon the bioavailability of AM; effectively, the rate (C_{max}) and extent ($AUC_{0\text{-}t}$) of absorption of AM enhanced by 3.8 and 2.4-fold, respectively, in healthy volunteers who received a single-dose of the drug immediately after consuming a high-fat meal *versus* following an overnight fast

(MENG *et al.,* 2001). The effects of food on the pharmacokinetics of AM were also studied in rats by SHAYEGANPOUR, JUN and BROCKS (2005) and the results obtained concerning the interference of lipids on the oral bioavailability of AM corroborated those reported in humans.

Nevertheless, in this case, taking into account the great content of soluble dietary fibre present in *F. vesiculosus*, we hypothesize the occurrence of a physical-chemical interaction between dietary fibre (or other extract components) and AM in the gastrointestinal tract to explain the considerable decrease in the systemic exposure/bioavailability of AM in the rats simultaneously co-administered in single-dose with *F. vesiculosus* extract. However, further studies are needed to understand the mechanism associated to the herb-drug interaction reported herein for the first time (*F. vesiculosus* extract/AM). At this point it should be highlighted the overview recently published by COLALTO (2010) about the herbal interactions on absorption of drugs and underlying interaction mechanisms. Dietary fibre may reduce the drug absorption, when both are assumed nearly, by a mechanism of action similar to bile sequestration; herb-drug interactions at absorption level associated with dietary fibre have been reported, as instance, for lovastatin (RICHTER, JACOB and SCHWANDT, 1991), digoxin (BROWN and JUHL, 1976; BROWN, JUHL and WARNER, 1978), metformin (GIN, ORGERIE and AUBERTIN, 1989) and glibenclamide (NEUGEBAUER, AKPAN and ABSHAGEN, 1983). In addition, LODEIRO *et al.* (2012) recently reported that *F. vesiculosus* interacts with aluminum of acidic waters by a mechanism of adsorption, suggesting that these physicochemical data may be of interest in modeling drug-food interactions, particularly those referring to aluminum-containing antacids-food pharmacokinetic process produced in the gastrointestinal tract. Therefore, similar physicochemical adsorption mechanisms may occur between *F. vesiculosus* and AM.

It is well-recognised the central role that CYP or P-glycoprotein (P-gp) induction or inhibition play on drug-drug and herb-drug interactions. In fact, AM is a substrate of P-gp (SHAPIRO and SHEAR, 2002) and is metabolized by several CYP isoenzymes in rat (ELSHERBINY, EL-KADI and BROCKS, 2010). Hence, to check the possible interference of *F. vesiculosus* extract on the CYP or P-gp activity, the extract was administered for 14 days (575 mg/kg/day, p.o.) until 24 h before applying AM; however, no significant influence was observed on the systemic (plasma) pharmacokinetics of AM in these circumstances. In these experimental conditions, it is only worthy of note the lower concentrations of AM in liver tissue, at 24 h post-dose, in rats pre-treated with *F. vesiculosus* extract (Figure IV.7).

Although this finding has not had in this case a significant impact on the magnitude of systemic exposition to AM, it is an interesting aspect to explore in further studies directed to evaluate the potential of enzyme induction by *F. vesiculosus.* Up to date, there is no evidence suggesting the hepatic metabolic induction of CYPs mediated by *F. vesiculosus*. On the contrary, the inhibition of CYP1A by trifucodiphlorethol A and trifucotriphlorethol A, compounds extracted from *F. vesiculosus*, was recently reported by PARYS *et al.* (2010).

Overall, considering the rat plasma data generated in the present work and that reported from clinical studies following oral administration of AM, it is evident that MDEA is the major metabolite of AM in both species, even though differences will exist in their metabolite-to-parent ratios. Indeed, in our pharmacokinetic studies, the plasma concentrations of MDEA found in rat were significantly lower than those of AM, and were found at levels near or below the LOQ (0.100 μg/mL) of the bioanalytical assay. Furthermore, AM and MDEA were found in concentrations considerably lower in plasma than in tissues (heart, liver, lungs and kidneys) at 24 h post-dose, supporting their great plasma/tissue distribution; these differences were absolutely remarkable for plasma/lung tissue. These rat tissues were selected for bioanalysis of AM and MDEA because they represent important targets from therapeutic (heart), toxicological (liver and lungs) and pharmacokinetic (liver and kidneys) viewpoints.

Based on data of herb-drug interaction between *F. vesiculosus* extract and AM, derived from this non-clinical investigation in rat, it is suggested that patients who are taking AM should avoid the concurrent administration of herbal slimming medicines/dietary supplements containing *F. vesiculosus.* In addition, in our experimental conditions, the sub-chronic administration of *F. vesiculosus* extract to rats (575 mg/kg/day, 14-day period, p.o.) not evidenced efficacy on weight reduction (Figure IV.8). It is also true that results from animal experiments cannot be directly extrapolated to humans; however, bearing in mind the studies of SHAYEGANPOUR, JUN and BROCKS (2005) and MENG *et al.* (2001) the rat appears to be an appropriate animal model for man in this situation.

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IV.7. SUPPLEMENTARY DATA

Certificate of analysis of bladderwrack *(Fucus vesiculosus L.)* 0.10% iodine dry aqueous extract provided by EPO Istituto Farmochimico Fitoterapico s.r.l. (N.: 201003344).

EPO ISTITUTO FARMOCHIMICO FITOTERAPICO s.r.l.

Via Stadera, 19 - 20141 Milano (Italy) - Tel. +39 02 89557.1 r.a. - Fax +39 0289557.291

CERTIFICATE OF ANALYSIS N.: 201003344

Bladderwrack 0.10 % dry aqueous extract

(Fucus vesiculosus L.)

COD.:	**3826801**
BATCH:	**1001553**
DATE OF PRODUCTION:	**06/09/2010**
EXPIRY DATE:	**06/09/2013**

Plant source:	**thallus**
EXCIPIENTS:	**maltodextrin (derived from maize) max 30.0%**

Manufacturer/Supplier: EPO S.r.l.	SPECIFICATIONS	RESULT
E/D Ratio:	up to stated assay % (1/3-4)	Complies
Description:	hygroscopic powder	Complies
Colour:	brown-rosy	Complies
Odor:	characteristic	Complies
Taste:	characteristic	Complies
Identification test:	complies (ref. Ph. Eur. current edition)	Complies
Assay: total Iodine (volumetric met.)	>= 0,10 % w/w	0,11 % w/w
Bulk density:	400 - 600 g/l	517 g/l
Loss on drying:	<= 5,0 % w/w	2,7 % w/w
pH (Sol.1:10):	4,5 - 7,0	5,2
Hydrosolubility:	partially watersoluble	Complies
Heavy metals:	< 20 ppm (method C Ph. Eur. current edition)	Complies
Lead:	< = 3.0 ppm	Complies
Cadmium:	< = 3.0 ppm	Complies
Mercury:	< = 0.10 ppm	Complies
Pesticides:	complies to Ph. Eur. current edition and Reg. 2005/396/EC and amendments	Complies
Aflatoxins:	Aflatoxin B1: < 2 ppb Aflatoxin B1,B2,G1,G2: < 4 ppb	Complies
Microbiological quality (Ref. Ph. Eur. current edition depending on the intended use)		
- Bacterial count (TAMC: ref. 5.1.8, cat. B oral use):	< = 5 x 10.000 ufc/g	1000 ufc/g
- Yeasts and Moulds (TYMC: ref. 5.1.8, cat. B oral use):	< = 5 x 100 ufc/g	< 50 ufc/g
- Pathogens (ref. 5.1.8, cat. B oral use):	Salmonella: absent in 25 g Escherichia coli: absent in 1 g	Complies
Bile-tolerant gram-negative bacteria (ref. 5. 1.8, cat. B oral use):	< = 100 ufc/g	Complies
Note:	GMO FREE (Reg. 1829/2003 - 1830/2003 EC) - GLUTEN FREE - BSE/TSE FREE this product does not contain food allergens (Annex IIIa Dir. 2007/68/EC)	Complies

Analytical methods from Ph. Eur. current edition, except w here differently specified

Storage: store in a well closed container away from moisture and direct sun light

Handling: handle in well ventilated room to avoid powder diffusion. Eliminate all ignition sources.

Date: 10/09/2010

Dott.ssa S.Vicentini
Technical Director

CHAPTER V

HERB-DRUG INTERACTION OF PAULLINIA CUPANA (GUARANA) SEED EXTRACT ON THE PHARMACOKINETICS OF AMIODARONE IN RATS

V. HERB-DRUG INTERACTION OF *PAULLINIA CUPANA* (GUARANA) SEED EXTRACT ON THE PHARMACOKINETICS OF AMIODARONE IN RATS

V.1. ABSTRACT

Paullinia cupana is used in weight-loss programs as a constituent of medicinal/dietary supplements. This study aimed to assess a potential herb-drug interaction among a standardized (certified) *P. cupana* extract and amiodarone (AM; narrow therapeutic index drug) in rats. In a first pharmacokinetic study rats were simultaneously co-administered with a single-dose of *P. cupana* (821 mg/kg, p.o.) and AM (50 mg/kg, p.o.), and in a second study rats were pre-treated during 14 days with *P. cupana* (821 mg/kg/day, p.o.) receiving AM (50 mg/kg, p.o.) on the 15th day. Rats of the control groups received the corresponding volume of vehicle. Blood samples were collected at several time points after AM dosing, and several tissues were harvested at the end of the experiments (24 h post-dose). Plasma and tissue concentrations of AM and its major metabolite (mono-*N*-desethylamiodarone) were measured and analysed. A significant reduction in the peak plasma concentration (73.2%) and in the extent of systemic exposure (57.8%) to AM was found in rats simultaneously treated with *P. cupana* and AM; a decrease in tissue concentrations was also observed. For the first time an herb-drug interaction between *P. cupana* extract and AM is reported, which determined a great decrease on AM bioavailability in rats.

V.2. INTRODUCTION

Paullinia cupana, commonly known as Guarana, is a climbing evergreen vine with small fruits and is native to the Amazon region (LIMA *et al.,* 2005; CAMPOS *et al.,* 2011). *P. cupana* seed extracts have been used in folk medicine since pre-Columbian times as stimulants, aphrodisiacs and tonics (CAMPOS *et al.,* 2011).

The seeds of *P. cupana* contain large amounts of methylxanthines (caffeine, theophylline and theobromine), saponins, and polyphenols, especially tannins, as well as trace concentrations of many other compounds (HEARD *et al.,* 2006; KREWER *et al.,* 2011; KLEIN, LONGHINI and DE MELLO, 2012). These constituents are probably responsible for the use of *P. cupana* seed extract in popular medicine as a stimulant of the central nervous system, in cases of physical and mental stress, and as an antidiarrheic, diuretic, and antineuralgic (KLEIN, LONGHINI and DE MELLO, 2012). Its high caffeine content and stimulating properties make *P. cupana* particularly attractive in weight-loss programs, since it helps increase the metabolic rate and can improve thermogenesis. Indeed, *P. cupana* (Guarana) containing products are popular among athletes because of their ergogenic and "fat burning" effects (BULKU *et al.,* 2012). Some studies have shown that *P. cupana* positively affects lipid metabolism (LIMA *et al.,* 2005), enhances weight loss (BOOZER *et al.,* 2001; OPALA *et al.,* 2006) and increases basal energy expenditure (BÉRUBÉ-PARENT *et al.,* 2005; KREWER *et al.,* 2011). Thus, these data suggest that *P. cupana* has anti-obesity effects (BOOZER *et al.,* 2001; BÉRUBÉ-PARENT *et al.,* 2005; OPALA *et al.,* 2006; KREWER *et al.,* 2011). Moreover, several studies have also ascribed to *P. cupana* antioxidant and cardioprotective effects (BYDLOWSKI, YUNKER and SUBBIAH, 1988; BYDLOWSKI, D'AMICO and CHAMONE, 1991; KREWER *et al.,* 2011; BULKU *et al.,* 2012).

P. cupana extract (from seed) is approved in the United States as a food additive and is considered a dietary supplement (HEARD *et al.,* 2006); *P. cupana* is also listed in the official Brazilian Pharmacopoeia (ANTONELLI-USHIROBIRA *et al.,* 2010). Therefore, it is not surprising that *P. cupana* (Guarana) is found today in a wide variety of drinks, foods, dietary/herbal supplements and pharmaceuticals (LIMA *et al.,* 2005; ANTONELLI-USHIROBIRA *et al.,* 2010). Consequently, perhaps concerned about the widespread use or potential abuse of *P. cupana* based products, the Committee on Herbal Medicinal Products (HMPC) of European Medicines Agency has recently launched a call to encourage submission of scientific data on *P. cupana* (Guarana) in order to prepare the corresponding herbal monograph (EUROPEAN MEDICINES AGENCY, 2011).

Given that obesity and overweight are increasing at an alarming rate in developed countries (PITTLER, SCHMIDT and ERNST, 2005), representing two of the major independent risk factors for cardiovascular diseases (SCAGLIONE *et al.,* 2004; BODARY, IGLAY and EITZMAN, 2007; ZALESIN *et al.,* 2011), a great increase on the consumption of dietary/herbal supplements containing *P. cupana* is still expected. Since the concurrent use of these products with conventional drugs may lead to significant clinical herb-drug interactions, it is urgent to assess the interference of *P. cupana* seed extract on the kinetics of narrow therapeutic index drugs, such as amiodarone (AM) (SINGH, 2008).

AM [2-*n*-butyl-3-(3,5-diiodo-4-diethylaminoethoxy-benzoyl)-benzofuran; (Figure V.1)] is one of the most widely prescribed antiarrhythmic agents for the treatment of atrial fibrillation and ventricular arrhythmias (PAPIRIS *et al.,* 2010). From a pharmacokinetic viewpoint AM has unusual and complex properties (SHAYEGANPOUR, HAMDY and BROCKS, 2008; VAN HERENDAEL and DORIAN, 2010), and it is recognised as a drug of narrow serum/plasma therapeutic range (0.5-2.0 μg/mL) (PÉREZ-RUIZ *et al.,* 2002; SHAYEGANPOUR, HAMDY and BROCKS, 2008). Furthermore, AM has also been associated to important clinical drug interactions (EDWIN, JENNINGS and KALUS, 2010; KARIMI *et al.,* 2010; ROUGHEAD *et al.,* 2010). Nevertheless, to the best of our knowledge, no study was conducted until now to evaluate herb-drug interactions between *P. cupana* (Guarana) and AM.

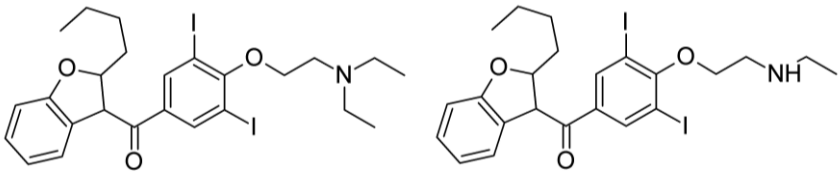

Figure V.1 – Chemical structures of amiodarone (AM) and its major metabolite mono-*N*-desethylamiodarone (MDEA).

Taking into account all the reasons previously referred and considering the potential for co-administration of *P. cupana* medicinal products and AM, this work was planned to investigate if a commercial standardized (certified) extract of *P. cupana* seeds may influence the pharmacokinetics of AM in rats, following their simultaneous oral co-administration and after a 14-day *P. cupana* pre-treatment period.

V.3. MATERIALS AND METHODS

V.3.1 DRUGS AND MATERIALS

Guarana *(P. cupana L.)* extract 12% caffeine obtained from fruit seeds was purchased from Bio Serae Laboratories (Bram, France); the corresponding certificate of analysis ref. 410044 (batch 0805519) is provided as *Supplementary Data.* Carboxymethylcellulose sodium salt for preparation of extract suspension was obtained from Sigma (St. Louis, MO, USA). A commercial formulation (ampoules) of AM hydrochloride 50 mg/mL solution for intravenous injection was used for oral administration to rats after appropriate dilution with 5% glucose intravenous solution for infusion (B. Braun Medical, Portugal). Other compounds used were sodium chloride 0.9% solution for injection (Labesfal, Portugal); heparin sodium 5000 U.I./mL for injection (B. Braun Medical, Portugal); ketamine for injection (Imalgene 1000) and xylazine for injection (Vetaxilaze 20). Introcan® Certo IV indwelling cannula (22G; 0.9 × 2.5 mm) made of polyurethane from B. Braun Melsungen AG (Melsungen, Germany).

V.3.2 ANIMALS

Adult male Wistar rats (310-380 g) of approximately 10 weeks old were obtained from local animal facilities (Faculty of Health Sciences of the University of Beira Interior, Covilhã, Portugal). The rats were maintained under controlled environmental conditions (temperature 20±2 °C; relative humidity 55±5%; 12-h light/dark cycle). The animals were allowed free access to a standard rodent diet (4RF21, Mucedola, Italy) during almost all experimental procedures and tap water was available *ad libitum*. At night on the day before dosing with AM, a lateral tail vein of each rat was cannulated, under anaesthesia [ketamine (90 mg/kg)/xylazine (10 mg/kg); i.p. injection], by insertion of an Introcan® Certo IV indwelling cannula (22G; 0.9 × 2.5 mm) used for serial blood sampling. The rats fully recovered from anaesthesia overnight and were fasted for 12-14 h before AM administration and maintained with free access to water; to avoid the effect of food on the oral bioavailability of AM an additional fasting period was considered (4 h post-dose). Oral treatments of the rats with *P. cupana* extract and AM were performed by gavage. Blood sampling was conducted in conscious and freely moving rats, which were appropriately restrained only at the moment of blood collection, except for the last blood sampling that

was taken by a terminal procedure (decapitation and exsanguination under anaesthesia). All the animal experiments were conducted in accordance with the European Directive (2010/63/EU) for the accommodation and care of laboratory animals and the experimental procedures were reviewed and approved by the Portuguese Veterinary General Division.

V.3.3 EXPERIMENTAL DESIGN AND PHARMACOKINETIC STUDIES

Two separate and independent pharmacokinetic studies were designed to investigate the effects of *P. cupana* on the kinetics of AM: (1) a single oral co-administration study of *P. cupana* extract and AM; and (2) a 14-day repeated oral pre-treatment study with *P. cupana* extract and on the 15th day a single oral dose of AM was given. The dose of *P. cupana* was selected based on the dose recommended to humans by the supplier of the extract (Bio Serae Laboratories) and taking into account the Food and Drug Administration (FDA) Guidance for Industry on conversion of animal doses to human equivalent doses (GUIDANCE FOR INDUSTRY, 2005); additionally, a 10-fold potentiating interaction factor was considered. On the other hand, the single oral dose of AM (50 mg/kg) was established because it has provided plasma concentrations of AM in rats within the plasma therapeutic range (SHAYEGANPOUR, JUN and BROCKS, 2005). In each day of the experiments *P. cupana* extract was suspended in 0.5% carboxymethylcellulose aqueous solution affording a suspension of herbal extract at 82.1 mg/mL. AM commercial injectable solution (50 mg/mL) was also appropriately diluted with 5% glucose solution to extemporaneously prepare an AM solution at 12.5 mg/mL. Appropriate volumes of *P. cupana* extract suspension (10 mL/kg of body weight) and of AM solution (4 mL/kg of body weight) were orally administered to rats by oral gavage.

In the first pharmacokinetic study, twelve Wistar rats were randomly divided into two groups (experimental and control groups). Rats of the experimental group (n = 6) were concomitantly treated with a single-dose of *P. cupana* extract (821 mg/kg, p.o.) and a single-dose of AM (50 mg/kg, p.o.); the extract suspension was administered right before AM. Rats of the control group (n = 6) received, instead of the *P. cupana* extract suspension, the corresponding volume of 0.5% carboxymethylcellulose aqueous solution (vehicle of the extract).

In the second pharmacokinetic study, twelve Wistar rats were also randomly divided into two groups. Rats assigned to the experimental group (n = 6) were orally pre-treated

with *P. cupana* extract (821 mg/kg, p.o.) once daily for 14 consecutive days (sub-chronic pre-treatment). Rats allocated to the control group (n = 6) were administered with an equivalent volume of vehicle for the same period of time. During the pre-treatment period, the rats were kept in 12-h light/dark cycle animal room with controlled temperature and humidity, as indicated above (*section V.3.2*); free access to a standard rodent diet and tap water was allowed. On 15th day, rats of both groups (experimental and control) were gavaged with a single-dose of AM (50 mg/kg, p.o.).

In both pharmacokinetic studies, the treatments with *P. cupana* extract (or vehicle) and/or AM were always carried out on the morning between 9:00 am and 11:45 am. At night on the day before AM administration, the rats were anaesthetized for cannulation of a lateral tail vein and were fasted overnight as described above *(section V.3.2).* On the day after, multiple serial blood samples (approximately 0.3 mL) were collected through the cannula into heparinized tubes before dosing and at 0.25, 0.5, 1, 2, 4, 6, 8 and 12 h following AM administration; then, at 24 h post-dose, blood and tissues (heart, liver, kidneys and lungs) were also harvested after decapitation of the rats. The blood samples were centrifuged at 4000 rpm for 10 min (4 °C) to separate the plasma which was stored at –20 °C until analysis. After exsanguination, liver, kidneys, heart and lungs were excised and stored at –20 °C; the organs were weighed and homogenized in distilled water (3 mL of water per gram of tissue) before analysis of tissue homogenates samples.

V.3.4 DRUG ANALYSIS

Plasma and tissue concentrations of AM and its main metabolite [mono-*N*-desethylamiodarone (MDEA)] were determined by using a liquid-liquid extraction (LLE) procedure followed by high-performance liquid chromatography-diode array detection (HPLC-DAD) assay previously developed and validated (RODRIGUES *et al.,* 2013). Briefly, an aliquot of each plasma sample (150 µL) was diluted with 150 µL of 0.1 M sodium phosphate buffer (pH 5) and spiked with 20 µL of the IS working solution (50 µg/mL). The mixture was added of 500 µL of *n*-hexane (used as LLE solvent), vortex-mixed for 30 sec and centrifuged at 17000 rpm for 2 min at 4 °C. The upper organic layer was transferred to a clean glass tube and the sample was re-extracted two more times with *n*-hexane (500 µL each time) using the same experimental conditions. Then, the whole organic extract was evaporated to dryness under a nitrogen stream at 60 °C and the residue was reconstituted in 100 µL of

methanol. Following this, an aliquot of the reconstituted extract (20 µL) was injected into the HPLC system for analysis.

For the extraction from tissues, each aliquot (400 µL) of tissue (heart, liver, kidney and lung) homogenates was spiked with 20 µL of the IS working solution (50 µg/mL); then, the mixture was added of 400 µL of acetonitrile (used as protein precipitating agent), vortex-mixed for 1 min and centrifuged at 17000 rpm for 10 min at 4 °C in order to precipitate the protein content. The supernatant was transferred to a new propylene tube and 1 mL of *n*-hexane (used as LLE solvent) was added. The mixture was vortex-mixed for 1 min and centrifuged at 17000 rpm for 5 min at 4 °C. The upper organic layer (*n*-hexane) was transferred to a clean glass tube and the sample was re-extracted two more times with *n*-hexane (0.8 mL each time) using the same conditions. The organic extract was evaporated to dryness, reconstituted, and then injected into the HPLC system using the same procedures as mentioned above for rat plasma samples. The limit of quantification (LOQ) was established at 0.100 µg/mL for AM and MDEA in plasma and in tissue homogenates.

V.3.5 PHARMACOKINETIC ANALYSIS

The plasma concentration *versus* time data for AM and MDEA obtained from each individual rat were submitted to a non-compartmental pharmacokinetic analysis using the WinNonlin® version 4.1 (Pharsight Co, Mountain View, CA, USA). The peak concentrations of AM and MDEA in plasma (C_{max}) and the time to reach C_{max} (t_{max}) were obtained directly from the experimental data. Other pharmacokinetic parameters estimated from the individual plasma concentration-time profiles were: area under the concentration-time curve (AUC) from time zero to the last sampling time at which concentrations were at or above the LOQ of the method (AUC_{0-t}), calculated by the linear trapezoidal rule; AUC from time zero to infinite ($AUC_{0-\infty}$), calculated from AUC_{0-t} + (C_{last}/k_{el}), where C_{last} is the last quantifiable concentration and k_{el} is the apparent terminal elimination rate constant calculated by log-linear regression of the terminal segment of the concentration-time profile; apparent terminal elimination half-life ($t_{1/2el}$) and mean residence time (MRT). The concentrations lower than the LOQ of the assay were taken as zero for all calculations.

V.3.6 EFFECT OF THE SUB-CHRONIC *PAULLINIA CUPANA* TREATMENT ON BODY WEIGHT

For the sub-chronic treatment study (a 14-day *P. cupana* treatment period), the body weight of the rats administered with *P. cupana* extract (821 mg/kg/day, p.o.; experimental group) or vehicle (control group) were adequately registered on the first day and on the last day (14^{th}) of these treatments in order to examine the effect of *P. cupana* extract on body weight changes.

V.3.7 STATISTICAL ANALYSIS

Data were reported as the mean ± standard error of the mean (SEM). Comparisons between two groups were usually performed using unpaired two-tailed Student's *t*-test; for body weight comparisons within the same group the paired Student's *t*-test was employed. A difference was considered to be statistically significant for a *p*-value lower than 0.05 ($p < 0.05$).

V.4. RESULTS

V.4.1 EFFECTS OF THE SIMULTANEOUS CO-ADMINISTRATION OF *PAULLINIA CUPANA* ON AMIODARONE PHARMACOKINETICS

The mean plasma concentration-time profiles (n = 6) of AM and its main metabolite (MDEA) obtained after intragastric co-administration of rats with a single-dose of *P. cupana* extract (821 mg/kg, p.o.) or vehicle (control group) and a single-dose of AM (50 mg/kg, p.o.) are shown in Figure V.2.

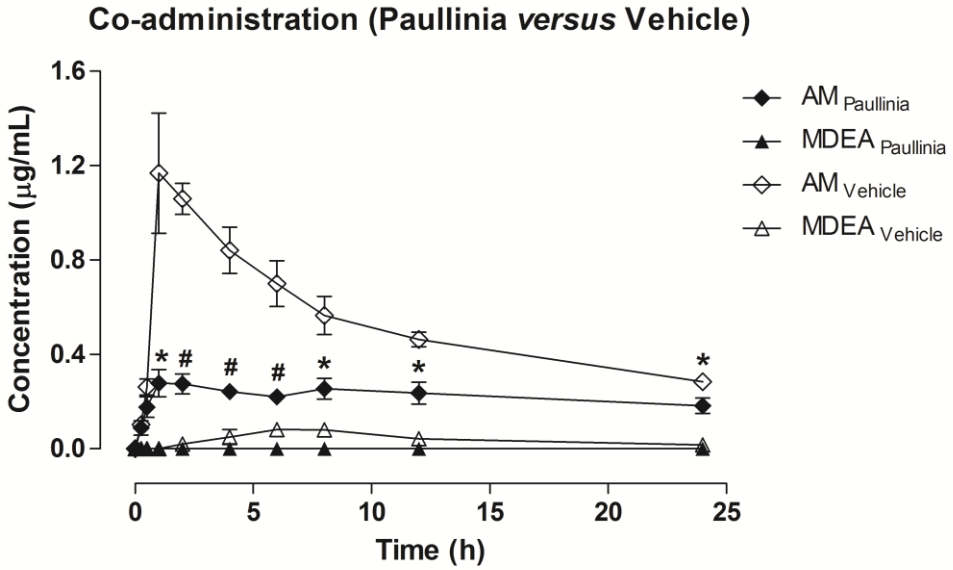

Figure V.2 – Mean plasma concentration-time profiles of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) obtained, over a period of 24 h, from rats simultaneously treated in single-dose with *Paullinia cupana* extract (821 mg/kg, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and AM (50 mg/kg, p.o.) by oral gavage. Symbols represent the mean values ± standard error of the mean (SEM) of six determinations per time point (n = 6). $^{*}p < 0.05$ and $^{\#}p < 0.001$ compared to control (vehicle).

AM plasma concentrations were similar in both groups only at the first two time-points post-dose (up to 0.5 h). AM plasma concentrations in the group treated with *P. cupana* extract were significantly lower than those in the control group over the 1-24 h post-dose time period (at least, $p < 0.05$). For MDEA, the plasma concentrations were only detected (not quantified) in the rats treated with *P. cupana* extract and in the rats of the control group the plasma concentrations were also low with values near or below the LOQ

(0.100 µg/mL). The main plasma pharmacokinetic parameters estimated for AM and MDEA after non-compartmental analysis of their concentration-time profiles are summarized in Table V.1.

Table V.1 – Pharmacokinetic parameters estimated by non-compartmental analysis of the plasma concentration-time profiles of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA, major metabolite of AM) obtained in rats after the simultaneous co-administration in single-dose of *Paullinia cupana* extract (821 mg/kg, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), with AM (50 mg/kg, p.o.) by oral gavage (n = 6, unless otherwise noted).

Parameter	$AM_{Paullinia}$		$AM_{Vehicle}$	
	AM	MDEA	AM	MDEA
t_{max} (h)	3.67 ± 1.17	NA	1.83 ± 0.48	7.20 ± 1.36[a]
C_{max} (µg/mL)	0.370 ± 0.043*	NA	1.378 ± 0.179	0.125 ± 0.012[a]
AUC_{0-t} (µ.h/mL)	5.387 ± 0.619*	ND	12.774 ± 0.688	ND
$AUC_{0-\infty}$ (µg.h/mL)	12.050 ± 2.118[#]	ND	21.431 ± 2.077	ND
k_{el} (1/h)	0.0310 ± 0.0044	ND	0.0433 ± 0.0082	ND
$t_{1/2el}$ (h)	24.18 ± 2.75	ND	20.73 ± 5.74	ND
MRT (h)	36.87 ± 4.28	ND	28.64 ± 7.74	ND

NA, not available; ND, not determined.
[a]n = 5.
*p < 0.001, significantly different from the control group.
[#]p < 0.05, significantly different from the control group.

With the co-administration of *P. cupana* extract the mean C_{max} of AM was significantly lower than that obtained in the control (vehicle) group (p < 0.001), while the mean time to reach C_{max} (t_{max}) was attained later in the experimental group (3.67 ± 1.17 h) comparatively to the control group (1.83 ± 0.48 h). Statistically significant differences were also observed for the AUC_{0-t} pharmacokinetic parameter (p < 0.001) calculated from the plasma concentration-time data obtained for AM in both groups; these differences were also evident for the $AUC_{0-\infty}$ parameter (p < 0.05) (Table V.1). Taking into consideration the information derived from Figure V.3, it is clear that following the simultaneous co-administration of *P. cupana* extract and AM a remarkable decrease (73.2%) in the C_{max} of the drug was observed, as well as a reduction of 57.8% in the extent of systemic drug exposure (as assessed by AUC_{0-t}).

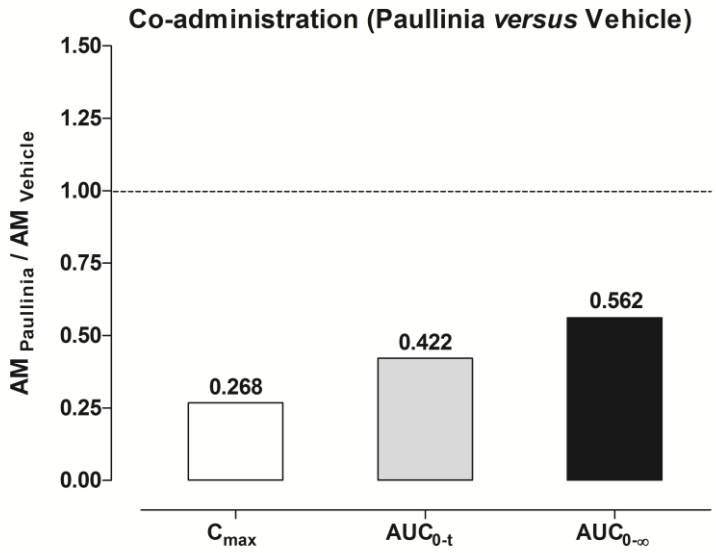

Figure V.3 – Ratios for the main plasma pharmacokinetic parameters (C_{max}, AUC_{0-t} and $AUC_{0-\infty}$) estimated for amiodarone (AM) in rats simultaneously treated in single-dose with *Paullinia cupana* extract (821 mg/kg, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and AM (50 mg/kg, p.o.) by oral gavage.

The mean values estimated for the elimination pharmacokinetic parameters are similar in both groups (*P. cupana* extract *versus* vehicle). Considering the paucity of quantifiable plasma concentrations obtained for MDEA, it was only possible to present the C_{max} and t_{max} parameters to the control (vehicle) group (Table V.1).

In addition, to examine some aspects related to the biodistribution of AM and MDEA in rats, both in the presence or absence of the co-administration with *P. cupana*, the animals were sacrificed at 24 h after dosing and several tissues were excised and analysed. The mean concentrations of AM and MDEA determined in heart, lung, liver and kidney tissues, and also their plasma concentrations at the same time (24 h) are shown in Figure V.4.

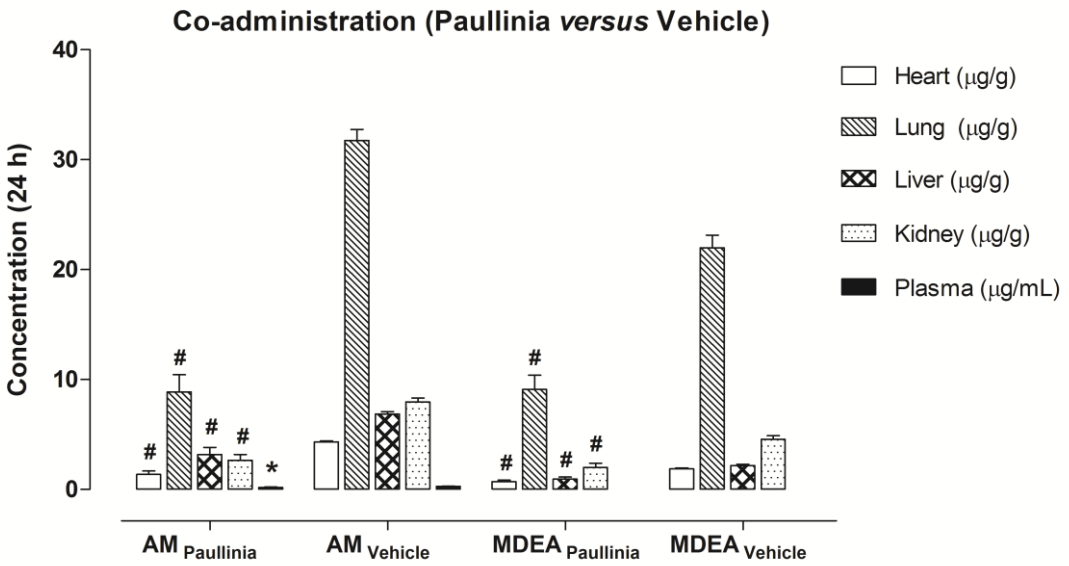

Figure V.4 – Mean plasma and tissue (heart, lung, liver and kidney) concentrations of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) obtained, at 24 h post-dose, from rats simultaneously treated in single-dose with *Paullinia cupana* extract (821 mg/kg, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and AM (50 mg/kg, p.o.) by oral gavage. Data are expressed as the mean values ± standard error of the mean (SEM) of six determinations ($n = 6$). $^{*}p < 0.05$ and $^{\#}p < 0.001$ compared to control (vehicle).

As indicated in Figure V.4, the tissue concentrations of AM and MDEA were markedly higher than those determined in plasma, and were absolutely noteworthy the levels found for both compounds (AM and MDEA) in the lung tissue. In addition, significant differences were found in the concentrations of AM and MDEA in tissues (heart, liver, kidney and lung) collected from experimental (*P. cupana* extract) and control (vehicle) groups ($p < 0.001$) at 24 h post-dose. As expected, the tissue concentrations in the group treated with *P. cupana* were lower than those measured in the control group, reflecting the differences observed in the extent of systemic drug exposure.

V.4.2 EFFECTS OF THE SUB-CHRONIC PRE-TREATMENT WITH *PAULLINIA CUPANA* ON AMIODARONE PHARMACOKINETICS

The rats were administered for 14 days with *P. cupana* extract (821 mg/kg, p.o.) or vehicle (control group) in order to investigate a possible interference of the *P. cupana* sub-chronic treatment on the pharmacokinetics of AM. The animals were administered with 50 mg/kg AM (p.o.) one day after the last treatment with *P. cupana* extract or vehicle, and the mean plasma concentration-time profiles (n = 6) of AM and its main metabolite (MDEA) are depicted in Figure V.5.

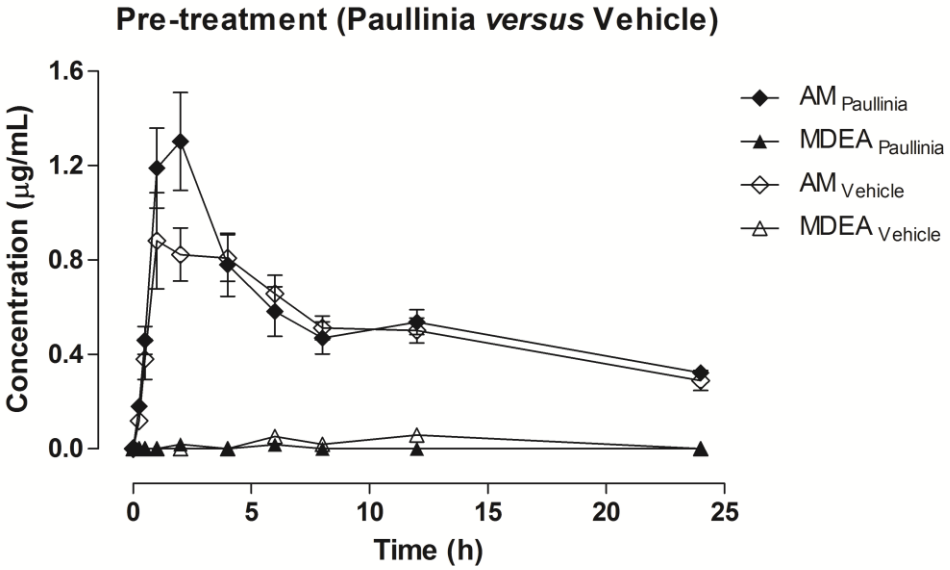

Figure V.5 – Mean plasma concentration-time profiles of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) obtained, over a period of 24 h, from rats submitted to a 14-day pre-treatment period with *Paullinia cupana* extract (821 mg/kg/day, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and treated on the 15th day with a single-dose of AM (50 mg/kg, p.o.) by oral gavage (n = 6). Symbols represent the mean values ± standard error of the mean (SEM) of six determinations per time point (n = 6).

The corresponding pharmacokinetic parameters, calculated by using non-compartmental analysis, are listed in Table V.2.

Table V.2 – Pharmacokinetic parameters estimated by non-compartmental analysis of the plasma concentration-time profiles of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA, major metabolite of AM) obtained in rats submitted to a 14-day pre-treatment period with *Paullinia cupana* extract (821 mg/kg/day, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and treated on the 15th day with a single-dose of AM (50 mg/kg, p.o.) by oral gavage (n = 6, unless otherwise noted).

Parameter	$AM_{Paullinia}$		$AM_{Vehicle}$	
	AM	MDEA	AM	MDEA
t_{max} (h)	1.50 ± 0.22	2.00 ± 0.00[a]	2.00 ± 0.63	9.00 ± 2.12[b]
C_{max} (µg/mL)	1.483 ± 0.203	0.112 ± 0.00[a]	1.046 ± 0.151	0.106 ± 0.004[b]
AUC_{0-t} (µg.h/mL)	13.422 ± 1.266	ND	12.282 ± 1.047	ND
$AUC_{0-\infty}$ (µg.h/mL)	23.489 ± 1.970	ND	22.057 ± 2.905	ND
k_{el} (1/h)	0.0416 ± 0.0079	ND	0.0442 ± 0.0108	ND
$t_{1/2el}$ (h)	23.07 ± 7.58	ND	21.62 ± 5.85	ND
MRT (h)	32.65 ± 10.41	ND	31.02 ± 8.29	ND

ND, not determined.
[a]n = 1; [b]n = 4.

Overall, it was observed a close overlap between the AM plasma pharmacokinetic profiles at the early absorption phase (up to 0.5 h) and at the elimination phase (4-24 h). AM plasma concentrations in the group treated with *P. cupana* were higher than those in the control group over the 1-2 h post-dose time period (Figure V.5). However, no significant differences (p > 0.05) in the pharmacokinetic parameters were detected for AM and its main metabolite (MDEA) among the two groups (*P. cupana versus* vehicle pre-treatment) (Table V.2). The plasma concentrations of MDEA were near or below the LOQ (0.100 µg/mL) of the method in both groups of rats. Regarding the data shown in Figure V.6, it appears that the magnitude of systemic exposure (as assessed by C_{max}) is slightly higher in the rats pre-treated with *P. cupana* extract, whereas the extent of systemic exposure to AM is similar among experimental and control groups (ratios near to unity; as assessed by AUC_{0-t} and $AUC_{0-\infty}$).

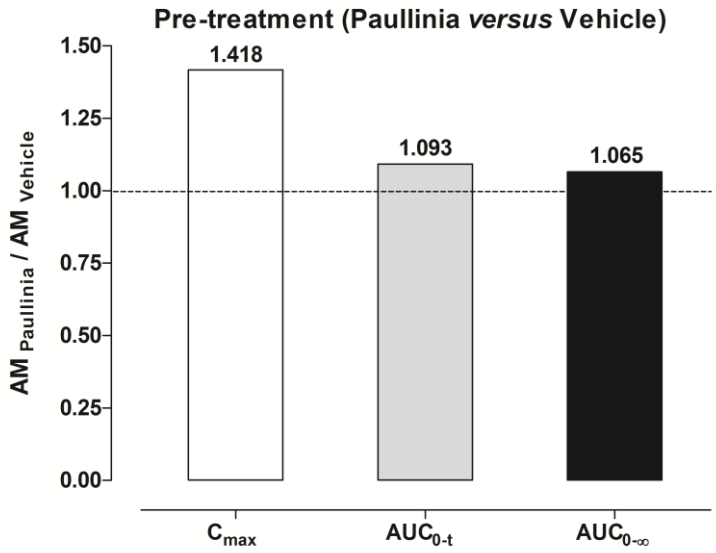

Figure V.6. – Ratios for the main plasma pharmacokinetic parameters (C_{max}, AUC_{0-t} and $AUC_{0-\infty}$) estimated for amiodarone (AM) in rats submitted to a 14-day pre-treatment period with *Paullinia cupana* extract (821 mg/kg/day, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and treated on the 15th day with a single-dose of AM (50 mg/kg, p.o.) by oral gavage.

To examine the influence of a 14-day pre-treatment period with *P. cupana* extract (experimental group) or vehicle (control group) on the distribution and metabolism of AM in rats, the concentrations of AM and its major metabolite (MDEA) were also determined in various tissues (additionally to plasma) at 24 h post-dose, and the data are shown in Figure V.7.

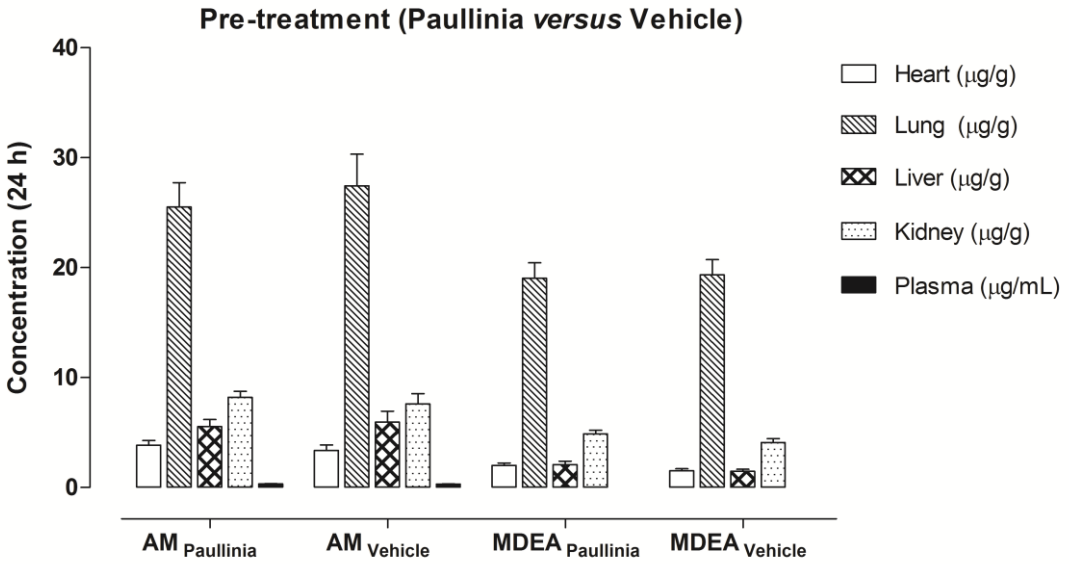

Figure V.7 – Mean plasma and tissue (heart, lung, liver and kidney) concentrations of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) obtained, at 24 h post-dose, from rats submitted to a 14-day pre-treatment period with *Paullinia cupana* extract (821 mg/kg/day, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and treated on the 15th day with a single-dose of AM (50 mg/kg, p.o.) by oral gavage. Data are expressed as the mean values ± standard error of the mean (SEM) of six determinations (*n* = 6).

As pointed out in Figure V.7, the concentrations of both compounds (AM and MDEA) in tissues were distinctly greater than those measured in plasma, and the concentration levels found in lung tissue were extremely high in experimental (*P. cupana*) and control (vehicle) groups. As it was expected from the plasma/systemic pharmacokinetic profiles obtained, no significant differences were detected in tissue concentrations between both groups.

V.4.3 EFFECT OF THE SUB-CHRONIC *PAULLINIA CUPANA* TREATMENT ON BODY WEIGHT

The resulting changes in body weight of the rats submitted to a 14-day treatment period with *P. cupana* extract (821 mg/kg/day, p.o) or vehicle are demonstrated in Figure V.8.

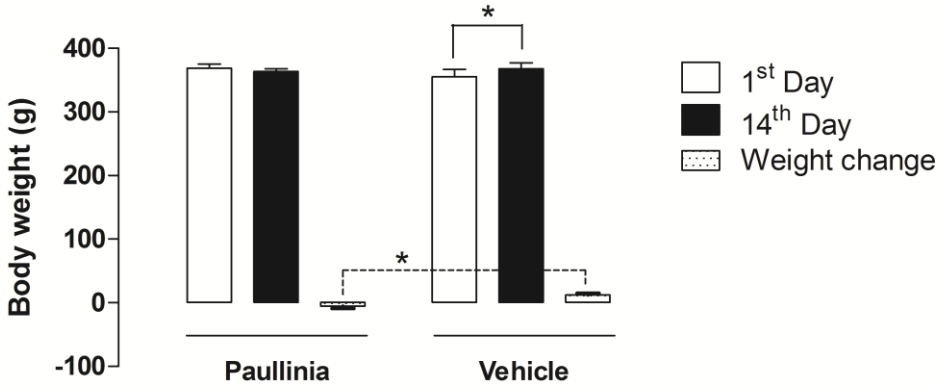

Figure V.8 – Effects on the body weight of the rats induced by the sub-chronic treatment (14-day period) with *Paullinia Cupana* extract (821 mg/kg/day, p.o.) and vehicle (0.5% carboxymethylcellulose aqueous solution) by oral gavage ($n = 6$). *$p < 0.01$.

From the analysis of the data it was found an increase statistically significant in body weight of rats treated with vehicle (control group) between the 1st and 14th day ($p < 0.01$), but differences were not observed in the group of rats administered with *P. cupana* extract ($p > 0.05$). On the other hand, by comparing the weight changes in both groups (*P. cupana* extract *versus* vehicle) a statistically significant difference was found, showing that the treatment with *P. cupana* extract was able to suppress gains in body weight of rats.

V.5. DISCUSSION

In literature, some interactions have been reported describing the interference of compounds, including herbal products, on the pharmacokinetics of AM. Between them, grapefruit juice can inhibit dramatically AM metabolism (LIBERSA *et al.,* 2000); in addition, orlistat can reduce significantly the systemic exposure to AM and MDEA (ZHI *et al.,* 2003); and, more recently, the exposure of rats to β-naphthoflavone (a polycyclic aromatic hydrocarbon) was found to increase the formation of MDEA probably through cytochrome P450 (CYP) induction (ELSHERBINY, EL-KADI and BROCKS, 2010). The present work was designed to investigate *in vivo* the potential of interaction between *P. cupana* extract and AM, using adult male Wistar rats (a whole-animal model). Taking into account that drug-drug or herb-drug interactions mainly occur at the level of absorption and/or metabolic (inhibition or induction) pathways, the pharmacokinetic studies reported herein were designed to examine the interference of *P. cupana* extract on the gastrointestinal absorption (simultaneous co-administration study) and on the metabolism of AM (14-day *P. cupana* pre-treatment study).

Our results clearly evidenced a significant decrease (73.2%) in the peak plasma concentration (C_{max}) of the drug following the simultaneous co-administration of the *P. cupana* extract and AM, as well as a reduction of 57.8% in the extent of systemic drug exposure (as assessed by AUC_{0-t}). On the other hand, an increase was also observed on the magnitude of systemic exposure to AM (as assessed by C_{max}) while no important differences were found in the extent of systemic drug exposure (as assessed by AUC_{0-t} and $AUC_{0-\infty}$) after the administration of the drug to pre-treated rats one day after the last treatment with *P. cupana* extract or vehicle. Hence, it is apparent that *P. cupana* extract or its components interact with AM in the gastrointestinal tract, reducing significantly the bioavailability of the drug after their simultaneous co-administration in a single-dose. However, the similarity observed in the extent of systemic exposure to AM in rats pre-treated for 14 days with *P. cupana* extract or vehicle excludes the impact of *P. cupana*-induced metabolism on the bioavailability of the drug. In fact, in the rats pre-treated for 14 days with *P. cupana* extract it was apparent an increasing of the magnitude of systemic exposure to AM but this difference was not statistically significant. Thus, the concomitant administration of *P. cupana* extract and AM in a single dose study supports the significance of the interaction at the level of the gastrointestinal tract. A significant reduction of the absorption of AM induced by orlistat in

healthy volunteers was also observed (ZHI *et al.,* 2003); this drug, a lipase inhibitor, significantly reduced the systemic exposure to AM by approximately 25% and a decrease of similar magnitude (~25%) was detected in the generation of the metabolite MDEA (the major metabolite of AM). According to ZHI *et al.* (2003) the absorption of highly lipophilic drugs such as AM may depend on the presence of a lipid phase in the gastrointestinal environment, which may be affected by the pharmacological action of orlistat. The magnitude (C_{max}) and extent (AUC_{0-t}) of absorption of AM was also enhanced in healthy volunteers who received a single-dose of the drug immediately after consuming a high-fat meal *versus* following an overnight fast (MENG *et al.,* 2001). SHAYEGANPOUR, JUN and BROCKS (2005) also studied the effects of food on the pharmacokinetics of AM in rats. The results obtained concerning the interference of lipids on the oral bioavailability of AM corroborated those reported in humans.

Taking into account the great diversity of components present in *P. cupana* extract, especially methylxantines (caffeine) and large quantity of tannins (CARLINI, 2003), we hypothesize the occurrence of a physical-chemical interaction between those compounds and AM in the gastrointestinal tract of rats to explain the considerable decrease in the systemic exposure/bioavailability of AM observed after the simultaneously co-administration with *P. cupana* extract. However, further studies are needed to understand the mechanism associated to this herb-drug interaction (*P. cupana* extract/AM), which is reported herein for the first time.

The central role that CYPs induction or inhibition plays on drug-drug and herb-drug interactions is well recognised. Hence, to check the possible interference of *P. cupana* extract on the CYP activity, the extract was administered for 14 days (821 mg/kg/day, p.o.) until 24 h before applying AM; however, only an apparent influence was observed on the magnitude of systemic (plasma) exposure to AM in these circumstances. Although this finding has not had in this case a significant impact on the magnitude of systemic exposure to AM, it is an interesting aspect to explore in further studies directed to evaluate the potential of enzyme induction or inhibition by *P. cupana*.

Globally, considering the rat plasma data generated in the present work and that reported from clinical studies following oral administration of AM, it is clear that MDEA is the major metabolite of AM in both species, despite differences will exist in their metabolite-to-parent ratios. Indeed, in our pharmacokinetic studies, the plasma concentrations of MDEA found in rat were significantly lower than those of AM, and were found at levels near

or below the LOQ (0.100 µg/mL) of the bioanalytical assay. Furthermore, AM and MDEA were found in concentrations considerably lower in plasma than in tissues (heart, liver, lungs and kidneys) at 24 h post-dose, supporting their great plasma/tissue distribution; these differences were absolutely remarkable for plasma/lung tissue. These rat tissues were selected for bioanalysis of AM and MDEA because they represent important targets from therapeutic (heart), toxicological (liver and lungs) and pharmacokinetic (liver and kidneys) viewpoints.

Based on the data of herb-drug interaction between *P. cupana* extract and AM derived from this non-clinical investigation in rat, it is suggested that patients who are taking AM should avoid the concurrent administration of herbal medicines/supplements containing *P. cupana.* However, taking also into account the absence of a significant herb-drug interaction between the extract and AM in the 14-day *P. cupana* pre-treatment study, it is not expected an important impact on the drug efficacy if medicinal products containing *P. cupana* extract and AM were administered separately in the time. It is also true that results from animal experiments cannot be directly extrapolated to humans however, bearing in mind the studies of SHAYEGANPOUR, JUN and BROCKS (2005) and MENG *et al.* (2001) the rat appears to be an appropriate animal model for man in this situation. Nevertheless, to reliably assess the clinical outcomes of the interaction between *P. cupana* extract and AM specific clinical trials are needed.

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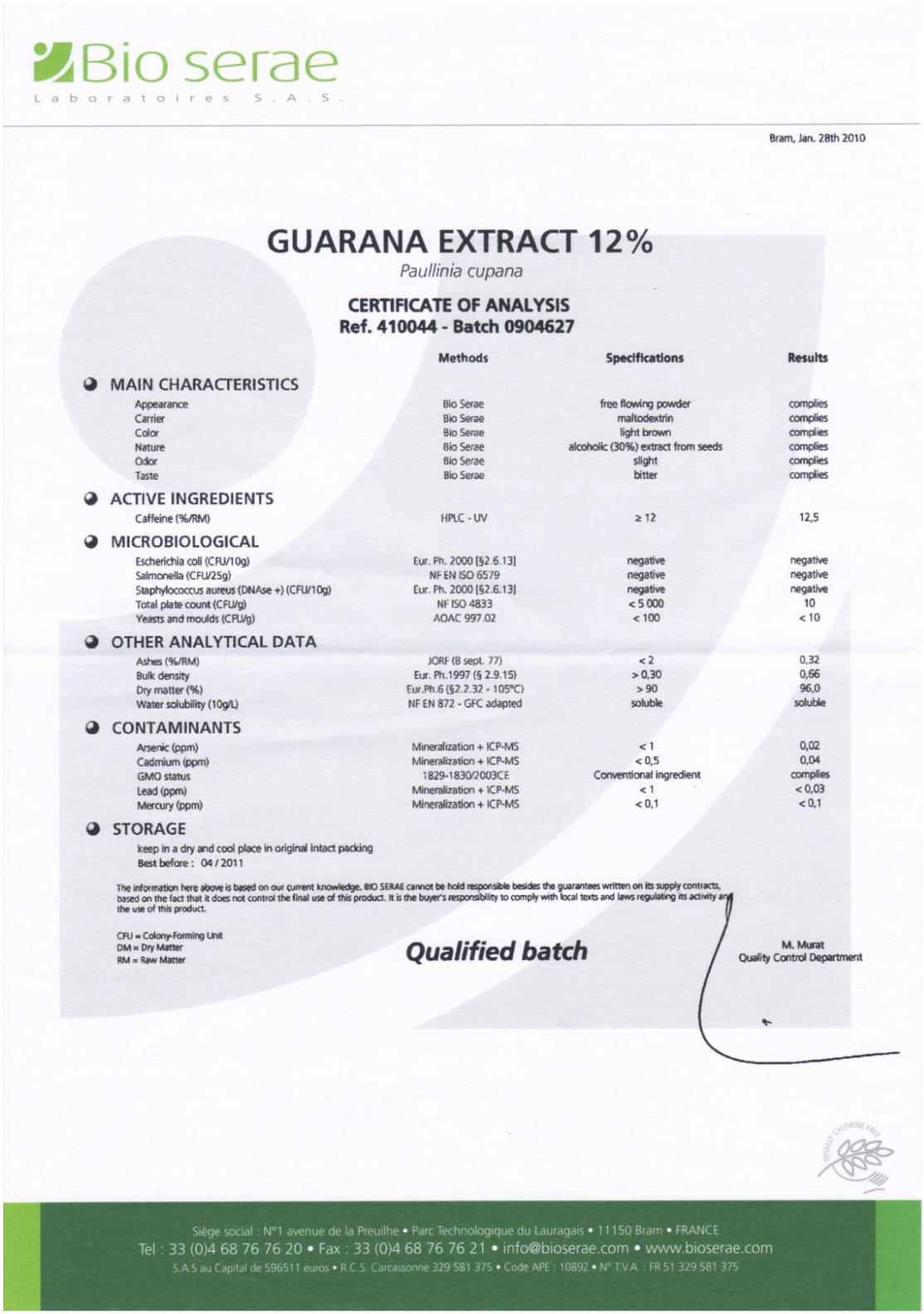
V.7. SUPPLEMENTARY DATA

Certificate of analysis of Guarana *(Paullinia cupana L.)* extract 12% caffeine provided by Bio Serae Laboratories (Ref. 41004 – Batch 0904627).



Bio serae
Laboratoires S.A.S.

Bram, Jan. 28th 2010

GUARANA EXTRACT 12%

Paullinia cupana

CERTIFICATE OF ANALYSIS
Ref. 410044 - Batch 0904627

	Methods	Specifications	Results
MAIN CHARACTERISTICS			
Appearance	Bio Serae	free flowing powder	complies
Carrier	Bio Serae	maltodextrin	complies
Color	Bio Serae	light brown	complies
Nature	Bio Serae	alcoholic (30%) extract from seeds	complies
Odor	Bio Serae	slight	complies
Taste	Bio Serae	bitter	complies
ACTIVE INGREDIENTS			
Caffeine (%/RM)	HPLC - UV	≥ 12	12,5
MICROBIOLOGICAL			
Escherichia coli (CFU/10g)	Eur. Ph. 2000 [§2.6.13]	negative	negative
Salmonella (CFU/25g)	NF EN ISO 6579	negative	negative
Staphylococcus aureus (DNAse +) (CFU/10g)	Eur. Ph. 2000 [§2.6.13]	negative	negative
Total plate count (CFU/g)	NF ISO 4833	< 5 000	10
Yeasts and moulds (CFU/g)	AOAC 997.02	< 100	< 10
OTHER ANALYTICAL DATA			
Ashes (%/RM)	JORF (8 sept. 77)	< 2	0,32
Bulk density	Eur. Ph.1997 (§ 2.9.15)	> 0,30	0,66
Dry matter (%)	Eur.Ph.6 (§2.2.32 - 105°C)	> 90	96,0
Water solubility (10g/L)	NF EN 872 - GFC adapted	soluble	soluble
CONTAMINANTS			
Arsenic (ppm)	Mineralization + ICP-MS	< 1	0,02
Cadmium (ppm)	Mineralization + ICP-MS	< 0,5	0,04
GMO status	1829-1830/2003CE	Conventional ingredient	complies
Lead (ppm)	Mineralization + ICP-MS	< 1	< 0,03
Mercury (ppm)	Mineralization + ICP-MS	< 0,1	< 0,1

STORAGE

keep in a dry and cool place in original intact packing
Best before : 04 / 2011

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CFU = Colony-Forming Unit
DM = Dry Matter
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CHAPTER VI

INVESTIGATING HERB-DRUG INTERACTIONS: THE EFFECT OF CITRUS AURANTIUM FRUIT EXTRACT ON THE PHARMACOKINETICS OF AMIODARONE IN RATS

VI. INVESTIGATING HERB-DRUG INTERACTIONS: THE EFFECT OF *CITRUS AURANTIUM* FRUIT EXTRACT ON THE PHARMACOKINETICS OF AMIODARONE IN RATS

VI.1. ABSTRACT

Citrus aurantium extract has been largely used in weight loss and sports performance dietary supplements. However, the safety of *C. aurantium*-containing products has been questioned mainly due to the association of its use with adverse events in the cardiovascular system. Therefore, this work aimed to assess the potential for herb-drug interactions among a standardized *C. aurantium* extract (GMP certificate) and amiodarone (AM; narrow therapeutic index drug) in rats. In a first pharmacokinetic study, rats were simultaneously co-administered with a single-dose of *C. aurantium* (164 mg/kg, p.o.) and AM (50 mg/kg, p.o.); in a second study, rats were pre-treated during 14 days with *C. aurantium* (164 mg/kg/day, p.o.) and received AM (50 mg/kg, p.o.) on the 15th day. Rats of the control groups received the corresponding volume of vehicle. Overall, after analysis of the pharmacokinetic data, it deserves to be highlighted the significant increase of the peak plasma concentration of AM in rats pre-treated with *C. aurantium* extract, while the extent of systemic exposure was comparable between both groups. For the first time, data on the potential of herb-drug interaction between *C. aurantium* extract and AM were reported. However, specific clinical trials should be performed to confirm these results in humans.

VI.2. INTRODUCTION

Citrus aurantium, previously called as *Fructus aurantii*, is the botanical name of a plant commonly known as bitter orange, sour orange, green orange, Seville orange or zhi shi (BOUCHARD *et al.,* 2005; HAAZ *et al.,* 2006). The extract of the immature fruit or peel of *C. aurantium* has been widely used in weight loss dietary supplements and in sports performance products (STOHS, PREUSS and SHARA, 2011a). In particular, after the ban of the sale of all ephedra-containing supplements by the Food and Drug Administration (FDA) in 2004, *C. aurantium* has gained an additional popularity as a safe alternative to *Ephedra* in herbal weight loss products (FOOD AND DRUG ADMINISTRATION and HHS, 2004; HANSEN *et al.,* 2012, 2013). *C. aurantium* has been used as an ingredient of the dietary supplements marketed for weight loss aid due to its claimed effects on metabolism, increasing the basal metabolic rate and lipolysis, and also as appetite suppressant (STOHS, PREUSS and SHARA, 2012). However, *C. aurantium* has not been traditionally employed for weight loss (HAAZ *et al.,* 2006). Historically, this herb has been mainly used in traditional Chinese medicine to treat gastrointestinal disorders like abdominal distension, dysentery and constipation (MATTOLI *et al.,* 2005; STOHS, PREUSS and SHARA, 2011a).

Synephrine, also called *p*-synephrine or oxedrine, is considered to be the main pharmacologically active protoalkaloid present in the extracts of immature fruit or peel of *C. aurantium*, which comprises more than 85% of the total protoalkaloid content. Additionally, other minor protoalkaloids constituents include the biogenic amines octopamine, hordenine, tyramine and *N*-methyltyramine (FUGH-BERMAN and MYERS, 2004; STOHS, PREUSS and SHARA, 2011a; HANSEN *et al.,* 2012). Structurally, synephrine is closely related to ephedrine (one of the main active constituents found in the genus *Ephedra*) (HANSEN *et al.,* 2012, 2013). However, despite their great structural similarities, synephrine contrary to ephedrine seems to exhibit little or no stimulant activity on the cardiovascular and central nervous system; the small chemical differences between synephrine and ephedrine also appear to significantly change their pharmacokinetic properties, particularly their ability to cross the blood-brain barrier (STOHS, PREUSS and SHARA, 2011b). Based on receptor binding studies, synephrine exhibited poor affinity for β-1, β-2 and α-adrenoreceptors, which are usually associated with cardiovascular effects (particularly β-1 and α-adrenoreceptors); instead, synephrine showed β-3 adrenergic activity which is responsible for increased thermogenesis and lipolysis (STOHS, PREUSS and SHARA, 2011a). Even so, the safety and efficacy of supplements containing *C.*

aurantium have been questioned (BENT, PADULA and NEUHAUS, 2004; FUGH-BERMAN and MYERS, 2004).

A large number of case reports have emerged over the last years associating the use of *C. aurantium*-containing products with serious clinical adverse events, most of them involving the cardiovascular system, such as syncope and prolongation of the QT interval (NASIR *et al.,* 2004), myocardial infarction (NYKAMP, FACKIH and COMPTON, 2004; THOMAS *et al.,* 2009), ischemic stroke (BOUCHARD *et al.,* 2005), angina (GANGE *et al.,* 2006) and tachycardia (FIRENZUOLI, GORI and GALAPAI, 2005), bradycardia and hypotension (GRAY and WOOLF, 2005), vasospasm and stroke (HOLMES JR. and TAVEE, 2008), ventricular fibrillation (STEPHENSEN and SARLAY JR., 2009) and ischemic colitis (SULTAN *et al.,* 2006). Furthermore, a pharmacokinetic herb-drug interaction involving a decoction of *C. aurantium* and cyclosporine was also reported (HOU *et al.,* 2000).

Hence, considering that *C. aurantium* has been extensively used as a replacement of *Ephedra* in the composition of weight loss dietary supplements and considering that the obesity and overweight are increasing at an alarming rate (PITTLER, SCHMIDT and ERNST, 2005), representing major independent risk factors for cardiovascular diseases (SCAGLIONE *et al.,* 2004; BODARY, IGLAY and EITZMAN, 2007; ZALESIN *et al.,* 2011), an increase on the consumption of herbal supplements containing *C. aurantium* is still expected. Thus, as the concurrent use of these herbal products and conventional drugs may lead to significant clinical herb-drug interactions, it is therefore absolutely pertinent to investigate the potential for pharmacokinetic interactions between *C. aurantium* and narrow therapeutic index drugs, as is the case of amiodarone (AM).

AM [2-*n*-butyl-3-(3,5-diiodo-4-diethylaminoethoxy-benzoyl)-benzofuran; (Figure VI.1)] still remains one of the most widely prescribed antiarrhythmic drugs for the treatment of atrial fibrillation and ventricular arrhythmias despite the availability of novel antiarrhythmic agents (PAPIRIS *et al.,* 2010). From a pharmacological viewpoint AM has unusual and complex pharmacokinetic properties (SHAYEGANPOUR, HAMDY and BROCKS, 2008; VAN HERENDAEL and DORIAN, 2010). AM has a variable oral bioavailability (20-80%) and the great lipophilicity of AM and its main metabolite [mono-*N*-desethylamiodarone (MDEA)] (Figure VI.1) leads to a high volume of distribution and a variable accumulation into tissues (VAN HERENDAEL AND DORIAN, 2010). MDEA is the product of the most predominant metabolic route of AM, the *N*-deethylation which is catalyzed by cytochrome P450 (CYP) isoenzymes (TRIVIER *et al.,* 1993; SOYAMA *et al.,* 2002). Moreover, AM is recognised as a drug of narrow therapeutic

window (0.5-2.0 µg/mL) (PÉREZ-RUIZ *et al.,* 2002; SHAYEGANPOUR, HAMDY and BROCKS, 2008) and it has also been associated to important clinical drug interactions (SIDDOWAY, 2003; EDWIN, JENNINGS and KALUS, 2010; KARIMI *et al.,* 2010; ROUGHEAD *et al.,* 2010).

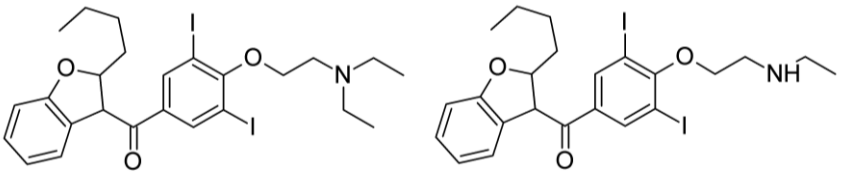

Figure VI.1 – Chemical structures of amiodarone (AM) and its major metabolite mono-*N*-desethylamiodarone (MDEA).

Taking into account all the reasons previously referred and bearing in mind the potential for co-administration of *C. aurantium* medicinal products and AM, this work was planned to investigate if a standardized extract of the green fruit of *C. aurantium* may influence the pharmacokinetics of AM in rats, following their simultaneous oral co-administration, and after a 14-day *C. aurantium* pre-treatment period.

VI.3. MATERIALS AND METHODS

VI.3.1 DRUGS AND MATERIALS

C. aurantium hidroalcoholic extract 10% synephrine (11.1% synephrine by high-performance liquid chromatography-ultraviolet detection batch analysis; approximately 94% of the total content in potent amines consists of synephrine) obtained from green fruit was purchased from Bio Serae Laboratories (Bram, France); the corresponding certificate of analysis ref. 410039 (batch 0907799) is provided as *Supplementary data*. Carboxymethylcellulose sodium salt for preparation of extract suspension was obtained from Sigma (St. Louis, MO, USA). A commercial formulation (ampoules) of AM hydrochloride 50 mg/mL solution for intravenous injection was used for oral administration to rats after appropriate dilution with 5% glucose intravenous solution for infusion (B. Braun Medical, Portugal). Other compounds used were sodium chloride 0.9% solution for injection (Labesfal, Portugal); heparin sodium 5000 U.I./mL for injection (B. Braun Medical, Portugal); ketamine for injection (Imalgene 1000) and xylazine for injection (Vetaxilaze 20). Introcan® Certo IV indwelling cannula (22G; 0.9 × 2.5 mm) made of polyurethane from B. Braun Melsungen AG (Melsungen, Germany).

VI.3.2 ANIMALS

Adult male Wistar rats (361 ± 26 g) of approximately 10 weeks old were obtained from local animal facilities (Faculty of Health Sciences of the University of Beira Interior, Covilhã, Portugal). The rats were maintained under controlled environmental conditions (temperature 20 ± 2 °C; relative humidity 55 ± 5%; 12-h light/dark cycle). The animals were allowed free access to a standard rodent diet (4RF21, Mucedola, Italy) during almost all experimental procedures and tap water was available *ad libitum*. At night on the day before dosing with AM, a lateral tail vein of each rat was cannulated, under anaesthesia [ketamine (90 mg/kg)/xylazine (10 mg/kg); i.p. injection], by insertion of an Introcan® Certo IV indwelling cannula (22G; 0.9 × 2.5 mm) used for serial blood sampling. The rats fully recovered from anaesthesia overnight and were fasted for 12-14 h before AM administration and maintained with free access to water; in order to avoid the effect of food on the oral bioavailability of AM an additional fasting period was considered (4 h post-dose). Oral

treatments of the rats with *C. aurantium* extract and AM were performed by gavage. Blood sampling was conducted in conscious and freely moving rats, which were appropriately restrained only at the moment of blood collection, except for the last blood sampling that was taken by a terminal procedure (decapitation and exsanguination under anaesthesia). All the animal experiments were conducted in accordance with the European Directive (2010/63/EU) for the accommodation and care of laboratory animals and the experimental procedures were reviewed and approved by the Portuguese Veterinary General Division.

VI.3.3 EXPERIMENTAL DESIGN AND PHARMACOKINETIC STUDIES

Two separate and independent pharmacokinetic studies were designed to investigate the effects of *C. aurantium* on the kinetics of AM: (1) a single oral co-administration study of *C. aurantium* extract and AM; and (2) a 14-day repeated oral pre-treatment study with *C. aurantium* extract and on the 15th day a single oral dose of AM was given. The dose of *C. aurantium* was selected based on the dose recommended to humans by the supplier of the extract (Bio Serae Laboratories) and taking into account the FDA Guidance for Industry on conversion of animal doses to human equivalent doses, which considers the body surface area (GUIDANCE FOR INDUSTRY, 2005); additionally, a 10-fold potentiating interaction factor was applied. The experimental dose of herbal extract selected is greater than the typical human daily dose to avoid false negative results and considering potential differences in the extrapolation between species (rat *versus* human). On the other hand, the single oral dose of AM (50 mg/kg) was established because it has provided plasma concentrations of AM in rats within the plasma therapeutic range (SHAYEGANPOUR, JUN and BROCKS, 2005). In each day of the experiments *C. aurantium* extract was suspended in 0.5% carboxymethylcellulose aqueous solution affording a suspension of herbal extract at 16.4 mg/mL. AM commercial injectable solution (50 mg/mL) was also appropriately diluted with 5% glucose solution to extemporaneously prepare an AM solution at 12.5 mg/mL. Appropriate volumes of *C. aurantium* extract suspension (10 mL/kg of body weight) and of AM solution (4 mL/kg of body weight) were orally administered to rats by oral gavage.

In the first pharmacokinetic study, twelve Wistar rats were randomly divided into two groups (experimental and control groups). The rats of the experimental group (n = 6) were concomitantly treated with a single-dose of *C. aurantium* extract (164 mg/kg, p.o.) and a single-dose of AM (50 mg/kg, p.o.); the extract suspension was administered just before

AM. The rats of the control group (n = 6) received, instead of the *C. aurantium* extract suspension, the corresponding volume of 0.5% carboxymethylcellulose aqueous solution (vehicle of the extract).

In the second pharmacokinetic study, twelve Wistar rats were also randomly divided into two groups. The rats assigned to the experimental group (n = 6) were orally pre-treated with *C. aurantium* extract (164 mg/kg, p.o.) once daily for 14 consecutive days (short-term repeated dose pre-treatment study), whereas the rats allocated to the control group (n = 6) were administered with an equivalent volume of vehicle for the same period of time. During the pre-treatment period, the rats were kept in 12-h light/dark cycle animal room with controlled temperature and humidity, as indicated above *(section VI.3.2);* free access to a standard rodent diet and tap water was allowed. On 15th day, the rats of both groups (experimental and control) were gavaged with a single-dose of AM (50 mg/kg, p.o.).

In both pharmacokinetic studies the treatments with *C. aurantium* extract (or vehicle) and/or AM were always carried out on the morning between 9:00 am and 11:45 am. At night on the day before AM administration, the rats were anaesthetized for cannulation of a lateral tail vein and were fasted overnight as described above *(section VI.3.2).* On the day after, multiple serial blood samples (approximately 0.3 mL) were collected through the cannula into heparinized tubes before dosing and at 0.25, 0.5, 1, 2, 4, 6, 8 and 12 h following AM administration; the blood samples were collected from all 6 rats in each treatment group at each time-point. Then, at 24 h post-dose, blood and tissues (heart, liver, kidneys and lungs) were also harvested after decapitation of the rats. The blood samples were centrifuged at 4000 rpm for 10 min (4 °C) to separate the plasma which was stored at –20 °C until analysis. After exsanguination, liver, kidneys, heart and lungs were excised and stored at –20 °C; the organs were weighed and homogenized in distilled water (3 mL of water per gram of tissue) before analysis of tissue homogenates samples.

VI.3.4 DRUG ANALYSIS

Plasma and tissue concentrations of AM and its main metabolite MDEA were determined by using a liquid-liquid extraction procedure coupled to the high-performance liquid chromatography-diode array detection assay previously developed and validated (RODRIGUES *et al.,* 2013a).

VI.3.5 PHARMACOKINETIC ANALYSIS

The plasma concentration *versus* time data for AM and MDEA obtained from each individual rat were submitted to a non-compartmental pharmacokinetic analysis using the WinNonlin® version 4.1 (Pharsight Co, Mountain View, CA, USA). The peak concentrations of AM and MDEA in plasma (C_{max}) and the time to reach C_{max} (t_{max}) were directly obtained from the experimental data. Other pharmacokinetic parameters estimated from the individual plasma concentration-time profiles were: area under the concentration-time curve (AUC) from time zero to the last sampling time at which concentrations were at or above the limit of quantification (LOQ; 0.100 µg/mL) of the method (AUC_{0-t}), calculated by the linear trapezoidal rule; AUC from time zero to infinite ($AUC_{0-\infty}$), calculated from AUC_{0-t} + (C_{last}/k_{el}), where C_{last} is the last quantifiable concentration and k_{el} is the apparent terminal elimination rate constant calculated by log-linear regression of the terminal segment of the concentration-time profile; apparent terminal elimination half-life ($t_{1/2el}$) and mean residence time (MRT). The concentrations lower than the LOQ of the assay were taken as zero for all calculations.

VI.3.6 SHORT-TERM REPEATED DOSE EFFECT OF *CITRUS AURANTIUM* ON BODY WEIGHT

In the pre-treatment study (a 14-day *C. aurantium* treatment period), the body weight of the rats administered with *C. aurantium* extract (164 mg/kg/day, p.o.; experimental group) or vehicle (control group) was adequately registered on the first day and on the last day (14^{th}) of the treatments in order to evaluate the effect of *C. aurantium* extract on body weight changes.

VI.3.7 STATISTICAL ANALYSIS

Data were reported as the mean ± standard error of the mean (SEM). Comparisons between two groups were usually performed using unpaired two-tailed Student's *t*-test; for body weight comparisons within the same group the paired Student's *t*-test was employed. A difference was considered to be statistically significant for a *p*-value lower than 0.05 ($p < 0.05$).

VI.4. RESULTS

VI.4.1 SIMULTANEOUS CO-ADMINISTRATION OF *CITRUS AURANTIUM* AND AMIODARONE

The mean plasma concentration-time profiles (n = 6) of AM and its main metabolite (MDEA) obtained after the intragastric simultaneous co-administration of rats with a single-dose of *C. aurantium* extract (164 mg/kg, p.o.) or vehicle (control group) and a single-dose of AM (50 mg/kg, p.o.) are shown in Figure VI.2.

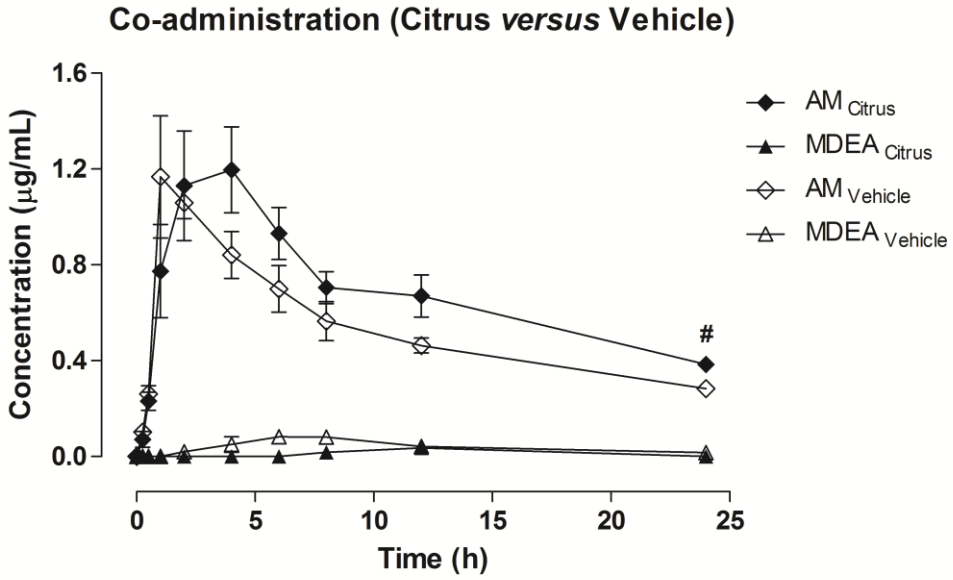

Figure VI.2 – Mean plasma concentration-time profiles of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) obtained, over a period of 24 h, from rats simultaneously treated in single-dose with *Citrus aurantium* extract (164 mg/kg, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and AM (50 mg/kg, p.o.) by oral gavage. Symbols represent the mean values ± standard error of the mean (SEM) of six determinations per time point (n = 6). $^{\#}p < 0.001$ compared to control (vehicle).

Overall, the mean plasma concentrations of AM were found to be statistically different only at 24 h post-dose ($p < 0.001$). In the case of MDEA, the plasma concentrations were similar in both groups, with values near or below the LOQ (0.100 μg/mL) of the method. The mean plasma pharmacokinetic parameters estimated for AM and MDEA after non-compartmental analysis of each individual concentration-time profile are summarized in Table VI.1.

Table VI.1 – Mean pharmacokinetic parameters estimated by non-compartmental analysis of the plasma concentration-time profiles of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA, major metabolite of AM) obtained in rats after the simultaneous co-administration in single-dose of *Citrus aurantium* extract (164 mg/kg, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), with AM (50 mg/kg, p.o.) by oral gavage (*n* = 6, unless otherwise noted).

Parameter	AM_{Citrus}		$AM_{Vehicle}$	
	AM	MDEA	AM	MDEA
t_{max} (h)	3.17 ± 0.75	12.00 ± 0.00[a]	1.83 ± 0.48	7.20 ± 1.36[b]
C_{max} (µg/mL)	1.405 ± 0.174	0.109 ± 0.006[a]	1.378 ± 0.179	0.125 ± 0.012[b]
AUC_{0-t} (µg.h/mL)	16.417 ± 1.553	ND	12.774 ± 0.688	ND
$AUC_{0-\infty}$ (µg.h/mL)	25.797 ± 1.185	ND	21.431 ± 2.077	ND
k_{el} (1/h)	0.0472 ± 0.0068	ND	0.0433 ± 0.0082	ND
$t_{1/2el}$ (h)	16.57 ± 2.78	ND	20.73 ± 5.74	ND
MRT (h)	24.63 ± 4.32	ND	28.64 ± 7.74	ND

ND, not determined.
[a]n = 2; [b]n = 5

From the observation of mean plasma concentration-time profiles of AM (Figure VI.2), it is evident that the time to reach C_{max} (t_{max}) was attained later in the experimental (*C. aurantium*) group than in the control (vehicle) group. Taking into consideration the information derived from Figure VI.3, it is clear that following the simultaneous co-administration of *C. aurantium* and AM, the magnitude of systemic exposure (as assessed by C_{max}) is similar among experimental and control groups and the extent of systemic exposure to AM (as assessed by AUC_{0-t} and $AUC_{0-\infty}$) is slightly higher in the rats co-administered with *C. aurantium* extract and AM.

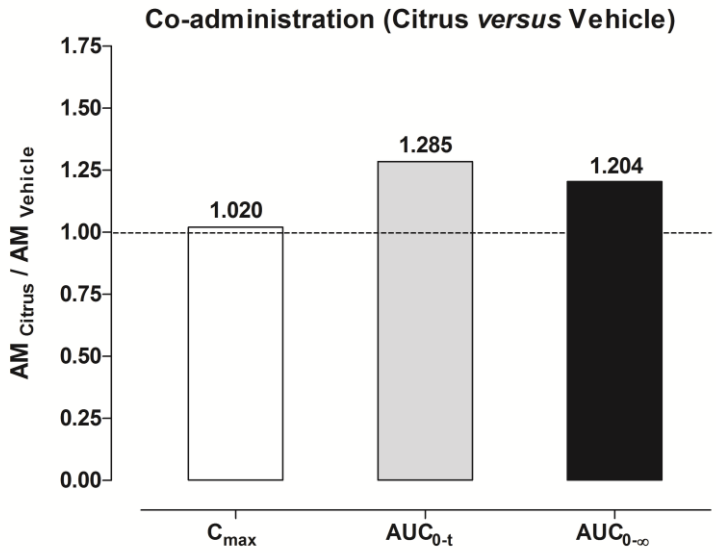

Figure VI.3 – Ratios for the main plasma pharmacokinetic parameters (C_{max}, AUC_{0-t} and $AUC_{0-\infty}$) estimated for amiodarone (AM) in rats simultaneously treated in single-dose with *Citrus aurantium* extract (164 mg/kg, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and AM (50 mg/kg, p.o.) by oral gavage.

However, no statistically significant differences in the mean pharmacokinetic parameters for AM were detected among the two groups (*C. aurantium versus* vehicle) (Table VI.1). These data show that the treatment of rats with *C. aurantium* extract does not significantly affect the extent of systemic exposure to AM (as assessed by AUC_{0-t} and $AUC_{0-\infty}$) despite the slight increase of these parameters in the group of rats co-administered with *C. aurantium* extract and AM, but the maximum exposure to the drug is delayed. Considering the scarcity of quantifiable plasma concentrations obtained for MDEA in both groups, only the C_{max} and t_{max} pharmacokinetic parameters are presented in Table VI.1.

To examine the biodistribution of AM and MDEA in rats, either in the presence or absence of the co-administration with *C. aurantium*, at 24 h after dosing all animals were sacrificed and several organs were excised and analysed. The mean concentrations of AM and MDEA determined in heart, lung, liver and kidney tissues, and also the plasma concentrations at the same time (24 h), are shown in Figure VI.4.

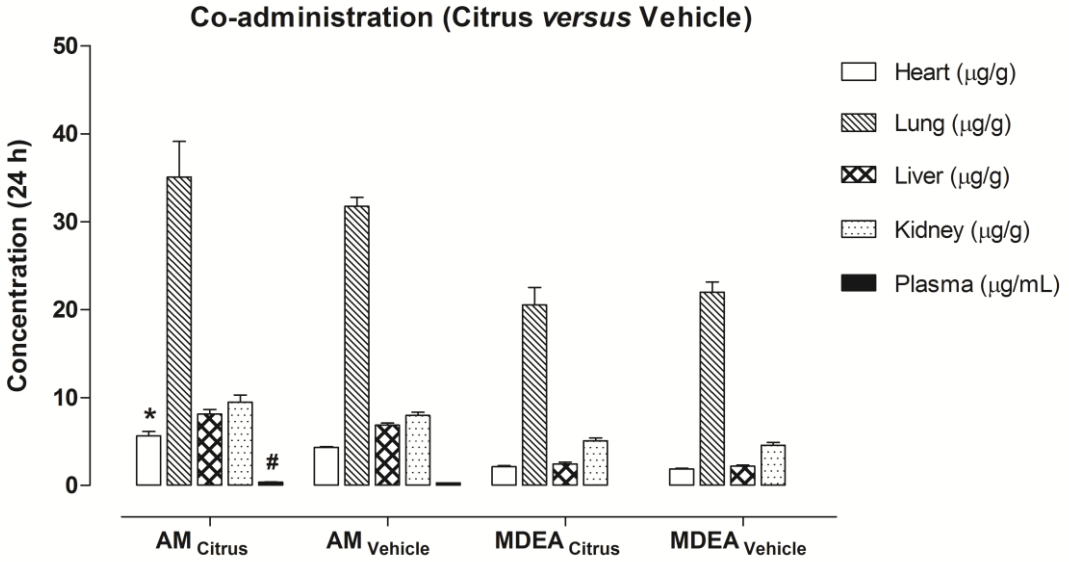

Figure VI.4 – Mean plasma and tissue (heart, lung, liver and kidney) concentrations of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) obtained, at 24 h post-dose, from rats simultaneously treated in single-dose with *Citrus aurantium* extract (164 mg/kg, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and AM (50 mg/kg, p.o.) by oral gavage. Data are expressed as the mean values ± standard error of the mean (SEM) of six determinations ($n = 6$). $^{*}p < 0.05$ and $^{\#}p < 0.001$ compared to control (vehicle).

The tissue concentrations of AM and MDEA were markedly higher than those measured in plasma, and the concentration levels found for both compounds (AM and MDEA) in the lung tissue were absolutely noteworthy. However, despite the significant differences detected at 24 h post-dose for AM plasma concentrations ($p < 0.001$), at the same time only for heart tissue were also found statistically significant differences ($p < 0.05$), and the concentrations of AM were higher in the group of rats treated with the extract.

VI.4.2 SHORT-TERM REPEATED DOSE PRE-TREATMENT STUDY WITH *CITRUS AURANTIUM* FOLLOWED BY AMIODARONE ADMINISTRATION

The rats were administered for 14 days with *C. aurantium* extract (164 mg/kg/day, p.o.; experimental group) or vehicle (control group) in order to investigate a possible interference of the short-term repeated dose pre-treatment with the extract on the pharmacokinetics of AM. The day after the last treatment with *C. aurantium* extract or vehicle, all animals received a single-oral dose of 50 mg/kg AM and the mean plasma

concentration-time profiles (n = 6) of the drug and its main metabolite (MDEA) are depicted in Figure VI.5.

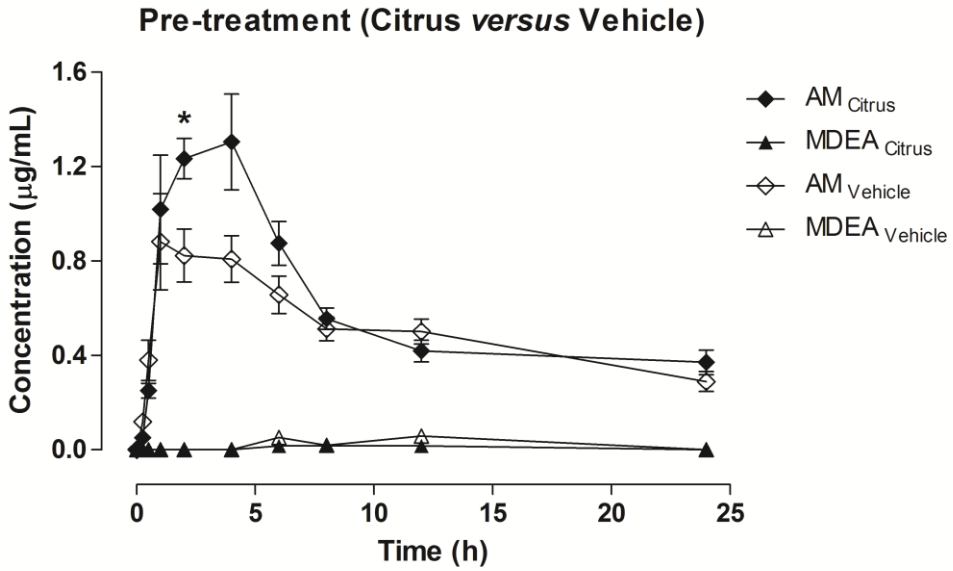

Figure VI.5 – Mean plasma concentration-time profiles of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) obtained, over a period of 24 h, from rats submitted to a 14-day pre-treatment period with *Citrus aurantium* extract (164 mg/kg/day, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and treated on the 15th day with a single-dose of AM (50 mg/kg, p.o.) by oral gavage (n = 6). Symbols represent the mean values ± standard error of the mean (SEM) of six determinations per time point (n = 6). *p < 0.05 compared to control (vehicle).

Comparing the mean plasma concentrations for AM in both groups, they were found to be statistically different only at 2 h post-dose (p < 0.05), and they were higher in the group treated with *C. aurantium*. The mean plasma pharmacokinetic parameters for AM and MDEA determined by applying non-compartmental analysis to each individual concentration-time profile are listed in Table VI.2.

Table VI.2 – Mean pharmacokinetic parameters estimated by non-compartmental analysis of the plasma concentration-time profiles of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA, major metabolite of AM) obtained in rats submitted to a 14-day pre-treatment period with *Citrus aurantium* extract (164 mg/kg/day, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and treated on the 15th day with a single-dose of AM (50 mg/kg, p.o.) by oral gavage (n = 6, unless otherwise noted).

Parameter	AM_{Citrus}		$AM_{Vehicle}$	
	AM	MDEA	AM	MDEA
t_{max} (h)	2.67 ± 0.61	7.00 ± 1.00[a]	2.00 ± 0.63	9.00 ± 2.12[b]
C_{max} (µg/mL)	1.654 ± 0.103*	0.107 ± 0.003[a]	1.046 ± 0.151	0.106 ± 0.004[b]
AUC_{0-t} (µg.h/mL)	14.318 ± 1.158	ND	12.282 ± 1.047	ND
$AUC_{0-\infty}$ (µg.h/mL)	23.901 ± 4.047	ND	22.057 ± 2.905	ND
k_{el} (1/h)	0.0485 ± 0.0074	ND	0.0442 ± 0.0108	ND
$t_{1/2el}$ (h)	16.38 ± 2.93	ND	21.62 ± 5.85	ND
MRT (h)	23.97 ± 3.89	ND	31.02 ± 8.29	ND

ND, not determined.
[a]n = 2; [b]n = 4.
*p < 0.01, significantly different from the control group.

The pre-treatment with *C. aurantium* extract determined a significantly higher C_{max} value for AM (p < 0.01), while for the other pharmacokinetic parameters no significant differences were found between both groups. The plasma concentrations of MDEA were near or below the LOQ (0.100 µg/mL) of the method in both groups. From the data shown in Figure VI.6, it is evident that the degree of systemic exposure to AM (as assessed by C_{max}) is higher in the rats pre-treated with *C. aurantium* extract comparatively with those of the control group, whereas the extent of systemic exposure to AM is similar among experimental and control groups (ratios near to unity; as assessed by AUC_{0-t} and $AUC_{0-\infty}$).

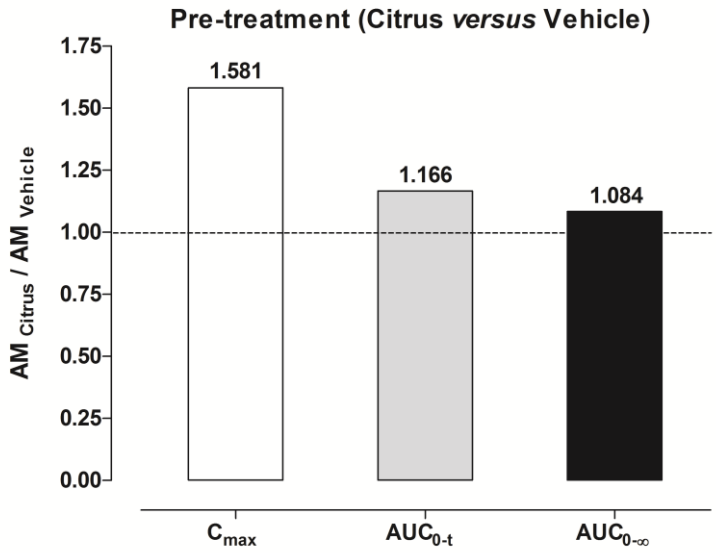

Figure VI.6 – Ratios for the main plasma pharmacokinetic parameters (C_{max}, AUC_{0-t} and $AUC_{0-\infty}$) estimated for amiodarone (AM) in rats submitted to a 14-day pre-treatment period with *Citrus aurantium* extract (164 mg/kg/day, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and treated on the 15th day with a single-dose of AM (50 mg/kg, p.o.) by oral gavage.

To investigate the influence of a 14-day pre-treatment period with *C. aurantium* extract (experimental group) or vehicle (control group) on the distribution and metabolism of AM in rats, the concentrations of AM and its major metabolite (MDEA) were also determined in various tissues (additionally to plasma) at 24 h post-dose and the results are shown in Figure VI.7.

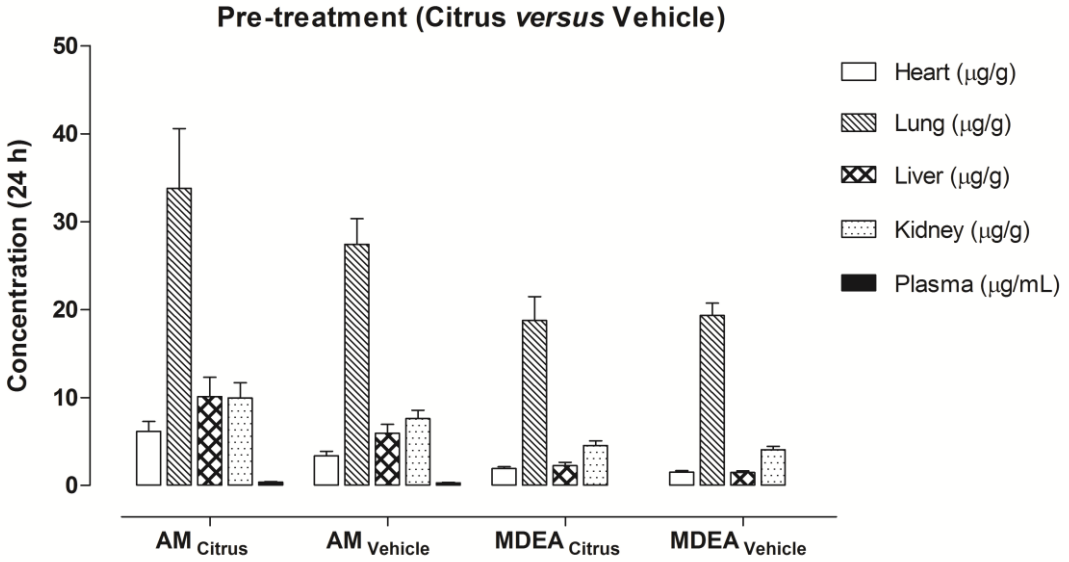

Figure VI.7 – Mean plasma and tissue (heart, lung, liver and kidney) concentrations of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) obtained, at 24 h post-dose, from rats submitted to a 14-day pre-treatment period with *Citrus aurantium* extract (164 mg/kg/day, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and treated on the 15th day with a single-dose of AM (50 mg/kg, p.o.) by oral gavage. Data are expressed as the mean values ± standard error of the mean (SEM) of six determinations (n = 6).

The concentrations of both compounds (AM and MDEA) in tissues were manifestly greater than those measured in plasma, and the concentration levels found in lung tissue were extremely high in both experimental (*C. aurantium*) and control (vehicle) groups. However, at 24 h post-dose, no significant differences were observed for AM and MDEA concentrations between both groups (AM_{Citrus} *versus* $AM_{Vehicle}$).

VI.4.3 SHORT-TERM REPEATED DOSE EFFECT OF *CITRUS AURANTIUM* ON BODY WEIGHT

The changes in body weight of the rats submitted to a 14-day pre-treatment period with *C. aurantium* extract (164 mg/kg/day, p.o) or vehicle are demonstrated in Figure VI.8.

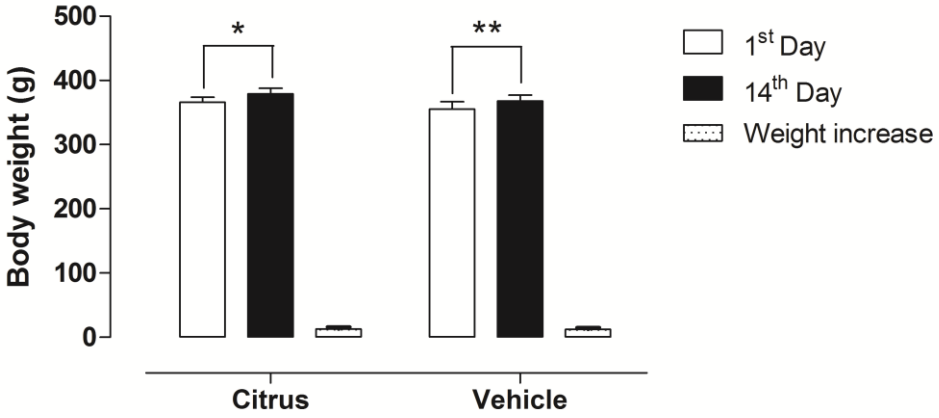

Figure VI.8 – Effects on the body weight of the rats induced by the short-term repeated dose treatment (14-day period) with *Citrus aurantium* extract (164 mg/kg/day, p.o.) and vehicle (0.5% carboxymethylcellulose aqueous solution) by oral gavage. *$p < 0.05$ and **$p < 0.01$, 1st day *versus* 14th day.

Statistically significant increases in body weight of rats treated either with *C. aurantium* extract ($p < 0.05$) or with vehicle ($p < 0.01$) between the 1st and 14th day of the experiments were observed. Furthermore, the increase in body weight of the rats of both groups (*C. aurantium versus* vehicle) was comparable. Hence, under these experimental conditions, the *C. aurantium* extract was shown to be ineffective to control the body weight gain in rats.

VI.5. DISCUSSION

Over the last years several interactions have been reported in literature describing the interference of herbal products and other compounds on the pharmacokinetics of AM. For instance, the grapefruit juice dramatically inhibited the metabolism of AM (LIBERSA *et al.,* 2000), the co-administration of orlistat and AM significantly reduced the systemic exposure to AM and its main metabolite MDEA (ZHI *et al.,* 2003), the exposure of rats to β-naphthoflavone (a polycyclic aromatic hydrocarbon) was found to increase the formation of MDEA probably through CYP induction (ELSHERBINY, EL-KADI and BROCKS, 2010) and, more recently, our research group documented the occurrence of herb-drug interactions between *Fucus vesiculosus* extract or *Paullinia cupana* extract and AM in rats (RODRIGUES *et al.,* 2012; RODRIGUES *et al.,* 2013b).

Accordingly, the present work was delineated to investigate the potential of interaction between *C. aurantium* extract and AM *in vivo*, using adult male Wistar rats. The pharmacokinetic studies herein reported were designed to examine the interference of *C. aurantium* extract on the gastrointestinal absorption (simultaneous co-administration study) and on the metabolism of AM (14-day *C. aurantium* pre-treatment study). In fact, drug-drug or herb-drug interactions mainly occur at the level of absorption process and/or metabolic (inhibition or induction) pathways.

Overall, our results show that the simultaneous co-administration of a single-dose of *C. aurantium* extract and AM caused an apparent delay to reach the peak plasma concentration of the drug, but it did not change significantly the magnitude and the extent of systemic exposure to AM (as assessed by C_{max} and AUC_{0-t} respectively). This increase in the time to achieve C_{max} is not expected to alter the efficacy of AM and it is unlikely to be clinically important. At this point, it deserves to be mentioned that after the co-administration of AM with other herbal extracts (*F. vesiculosus* extract and *P. cupana* extract), which are also claimed to be useful for weight loss, a delay in the time to reach C_{max} was also observed; however, in these studies a significant decrease in the systemic exposure to AM was clearly evident in the rats treated with herbal extracts (RODRIGUES *et al.,* 2012; RODRIGUES *et al.,* 2013b).

Moreover, because of the central role that the induction of CYPs and P-glycoprotein (P-gp) plays on drug-drug and herb-drug interactions, and bearing in mind that the induction mechanisms are time-dependent, the interference of *C. aurantium* extract on the

pharmacokinetics of AM was also investigated by administering the extract for 14 consecutive days (164 mg/kg/day, p.o.) until 24 h before applying AM. In fact, AM is metabolized by several CYP isoenzymes including CYP1A1/2, CYP2C8, CYP2C19, CYP2D6 and CYP3A4 (OHYAMA *et al.,* 2000; ELSHERBINY, EL-KADI and BROCKS, 2010) and is a substrate of P-gp (SHAPIRO and SHEAR, 2002; KALITSKY-SZIRTES *et al.,* 2004). The only significant change was an increase in the level of systemic exposure to AM (as assessed by C_{max}) in the rats pre-treated during 14 days with *C. aurantium* extract (*versus* vehicle). No significant differences were found in the extent of systemic drug exposure (as assessed by AUC_{0-t} and $AUC_{0-\infty}$). Therefore, to explain the increase of the C_{max} in the group of rats pre-treated with *C. aurantium* we hypothesise that the extract or some of its constituents could change (increase) the gastrointestinal motility, thus increasing the rate at which AM passes to the intestine. Actually, the absorption of AM across the intestinal membrane occurs by passive diffusion, which is a non-saturable process (MARTÍN-ALGARRA *et al.,* 1997). According to the studies of JINZHAO *et al.* (2005), the *C. aurantium* may improve the gastric emptying in rats with functional dyspepsia. Furthermore, extracts of *C. aurantium* also increased the rate of gastrointestinal motility enhancing, therefore, the absorption function of gastrointestinal tract (LI *et al.,* 2007). In addition, *Citrus aurantium* can also antagonise the inhibition of the intestinal advance induced by atropine (XUE-BAO *et al.,* 2005). In a study conducted by FANG *et al.* (2009) the effects of constituents of *C. aurantium* on the gastrointestinal movement were evaluated; the hesperidin had a stimulatory effect on the gastrointestinal muscle contraction while synephrine had an inhibitory effect.

Another possibility to explain the higher C_{max} values for AM in the group of rats pre-treated with *C. aurantium* extract could be related to a time-dependent inhibitory effects induced by *C. aurantium* or some of its phytochemicals on the CYPs and/or P-gp–mediated efflux activity. Actually, in a non-clinical study was reported that a decoction of *C. aurantium* increased the bioavailability of cyclosporine (HOU *et al.,* 2000).

Based on the herb-drug interaction data obtained in the present work involving *C. aurantium* and AM, it is suggested that the tested *C. aurantium* extract has no significant impact on the pharmacokinetic of AM, even using an experimental dose of extract in rats higher than the typical recommended dose in humans. Even so, it should be taken in account that results from animal experiments cannot be directly extrapolated to humans; however, bearing in mind the studies of SHAYEGANPOUR, JUN and BROCKS (2005) and MENG *et al.* (2001), the rat appears to be an appropriate animal model for man in this case.

Nevertheless, to reliably assess the safety of the administration of *C. aurantium* extract and AM specific clinical trials are required.

Additionally, in our study the increase of body weight of the rats pre-treated with *C. aurantium* extract or vehicle was comparable. These data are in accordance with other results found in the literature for *C. aurantium* extract. ARBO *et al.* (2008) also reported that the body weight gain did not change significantly comparing with the control group in rats treated during 28 days with 400, 2000 and 4000 mg/kg of *C. aurantium* extract. Only was observed a reduction in the gain of body weight for rats treated with 30 and 300 mg/kg of synephrine during 28 days (ARBO *et al.,* 2008).

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VI.7. SUPPLEMENTARY DATA

Certificate of analysis of *Citrus aurantium* extract 10% synephrine provided by Bio Serae Laboratories (Ref. 410039 – Batch 0907799).

Bio serae
Laboratoires S.A.S.

Bram, Sep. 7th 2009

CITRUS AURANTIUM EXTRACT 10%

Citrus aurantium

CERTIFICATE OF ANALYSIS
Ref. 410039 - Batch 0907799

	Methods	Specifications	Results
MAIN CHARACTERISTICS			
Appearance	Bio Serae	free flowing powder	complies
Color	Bio Serae	light brown	complies
Nature	Bio Serae	hydroalcoholic extract from fruit	complies
Odor	Bio Serae	characteristic of the plant	complies
Taste	Bio Serae	characteristic of the plant	complies
ACTIVE INGREDIENTS			
Potent amines (total) (%/RM)	HPLC-UV	≥ 11	11,8
Synephrine (%/RM)	HPLC-UV	≥ 10	11,1
MICROBIOLOGICAL			
Escherichia coli (CFU/10g)	Eur. Ph. 2000 [§2.6.13]	negative	negative
Staphylococcus aureus (DNAse +) (CFU/10g)	Eur. Ph. 2000 [§2.6.13]	negative	negative
Total plate count (CFU/g)	NF ISO 4833	< 10 000	100
Yeasts and moulds (CFU/g)	AOAC 997.02	< 500	< 100
OTHER ANALYTICAL DATA			
Apparent density (bulk & tap)	Eur. Ph.1997 (§ 2.9.15)	> 0,40	0,43 / 0,60
Ashes (%/RM)	JORF (8 sept. 77)	< 10	1,4
Dry matter (%)	Eur.Ph.6 (§2.2.32 - 105°C)	> 90	98,1
CONTAMINANTS			
Allergens free guarantee	Dir. 2003/89/CE and 2006/142	conform	complies
Arsenic (ppm)	Mineralization + ICP-MS	< 2	< 0,5
Benzo[a]pyrene (ppb)	NF EN ISO 17993	< 3.8	< 0,32
Cadmium (ppm)	Mineralization + ICP-MS	< 1	< 0,01
GMO status	1829-1830/2003CE	Conventional ingredient	complies
Lead (ppm)	Mineralization + ICP-MS	< 3	< 0,1
Mercury (ppm)	Mineralization + ICP-MS	< 0,1	< 0,005
STORAGE			
keep in a dry and cool place in original intact packing			
Best before : 06 / 2011			

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CHAPTER VII

HERB-DRUG PHARMACOKINETIC INTERACTION BETWEEN CARICA PAPAYA EXTRACT AND AMIODARONE IN RATS

VII. HERB-DRUG PHARMACOKINETIC INTERACTION BETWEEN *CARICA PAPAYA* EXTRACT AND AMIODARONE IN RATS

VII.1. ABSTRACT

Carica papaya extracts have been used for various ailments in humans, including obesity. However, scientific information is lacking on the potential for herb-drug interactions involving *C. papaya* and conventional drugs. Thus, this work aimed to investigate the interferences of a standardized *C. papaya* extract (GMP certificate) on the systemic exposure of amiodarone (AM; a narrow therapeutic index drug) in rats. In the first pharmacokinetic study, rats were simultaneously co-administered with a single-dose of *C. papaya* (1230 mg/kg, p.o.) and AM (50 mg/kg, p.o.); in the second study, rats were pre-treated for 14 days with *C. papaya* (1230 mg/kg/day, p.o.) and received AM (50 mg/kg, p.o.) on the 15th day. Rats of the control groups received the herbal extract vehicle. Despite the delay observed in both studies in attaining the maximum plasma concentrations of AM in the rats treated with the extract, the marked increase found in the extent of systemic exposure to AM (60-70%) in the rats pre-treated with *C. papaya* must be highlighted. For the first time, an herb-drug interaction between *C. papaya* extract and AM was reported, which clearly increase the drug bioavailability. To reliably assess the clinical impact of these findings appropriate clinical trials should be conducted.

VII.2. INTRODUCTION

Carica papaya, also traditionally known as pawpaw or papaya, is a tree-like herbaceous plant belonging to the family of Caricaceae (CANINI *et al.,* 2007; SADEK *et al.,* 2012). Mainly by its edible fruits, *C. papaya* is widely cultivated in several tropical, sub-tropical and temperate regions, including Australia, Brazil, China, Hawaii, Malaysia and India (CANINI *et al.,* 2007; AFZAN *et al.,* 2012). Different parts of the plant (fruits, leaves, barks, roots, flowers, seeds, and latex) as well as some of their extracts have been traditionally used worldwide in folk medicine to treat a wide range of ailments in humans (ANUAR *et al.,* 2008; NGUYEN *et al.,* 2013).

The fruits of *C. papaya* are one of the most commonly consumed throughout the world (ATHESH *et al.,* 2012), constituting a nutritional source rich in fibre, minerals and antioxidant nutrients (SADEK *et al.,* 2012). More specifically, papaya fruit is a good source of bioactive phytochemicals such as carotenoids (β-carotene, α-carotene, β-cryptoxanthin, α-cryptoxanthin, lutein, 9-*cis*-β-carotene), phenolic compounds (ferulic acid, caffeic acid, *p*-coumaric acid, rutin, quercetin, kaempferol), and glucosinolates (benzyl glucosinolate, benzyl isothiocyanate) (NGUYEN *et al.,* 2013). Unripe pulp of *C. papaya* also contains cardenolides which seem to have medicinal value for the treatment of congestive heart failure (ANUAR *et al.,* 2008). Additionally, among other biologically active constituents, papaya also includes cysteine proteinases as chymopapain and papain (KOVENDAN *et al.,* 2012; SADEK *et al.,* 2012) and its biological activity is often analysed through the proteolytic activity, particularly the papain activity (ANUAR *et al.,* 2008).

The extracts of ripe fruits are used for a variety of medicinal purposes including treatment of ringworm, malaria, hypertension (SADEK *et al.,* 2012), whereas extracts of unripe fruits have been used in the treatment of ulcers (EZIKE *et al.,* 2009) and diabetes (SADEK *et al.,* 2012). In addition, the hypoglycemic and hypolipidemic effects of the aqueous seed extract of *C. papaya* in rats have been reported (ADENEYE and OLAGUNJU, 2009). The ethanolic extract and water-soluble fraction of *C. papaya* also showed anti-hyperlipidemic activity in olive-treated rats (IVER *et al.,* 2011). More recently, ATHESH *et al.* (2012) reported the anti-obesity effect of aqueous fruit extract of *C. papaya* in rats fed on high fat cafeteria diet. Effectively, besides the proteolytic enzymes, chymopapain and papain, *C. papaya* also has a lipase enzyme that can contribute to its lipolytic action, supporting its use in the

management of obesity and digestive disorders (IZZO, 2004; KOVENDAN *et al.,* 2012; SADEK *et al.,* 2012).

Despite people have used medicinal herbs since ancient times (CALIXTO, 2000), a dramatic increase in the use of herbal products around the world has been observed in recent years (BENT, 2008). As consequence, there is an increased risk for herb-drug interactions because the phytochemicals are handled in the body through the same type of mechanisms involved in drug biodisposition (VENKATARAMANAN, KOMOROSKI and STROM, 2006). Importantly, *C. papaya* extract was associated to an increase of the international normalized ratio (INR) in a patient taking warfarin concomitantly (SHAW *et al.,* 1997). Hence, it has been suggested that *C. papaya* is contraindicated in patients receiving warfarin therapy (SHAW *et al.,* 1997; NUTESCU *et al.,* 2006).

Bearing in mind that obesity and overweight are increasing at an alarming rate worldwide (PITTLER, SCHMIDT and ERNST, 2005), representing major independent risk factors for cardiovascular diseases (SCAGLIONE *et al.,* 2004; BODARY, IGLAY and EITZMAN, 2007; ZALESIN *et al.,* 2011), and considering the anti-obesity effects recently reported for the aqueous fruit extract of *C. papaya* (ATHESH *et al.,* 2012), an increase in the consumption of herbal supplements containing *C. papaya* is still expected. Thus, as the occurrence of herb-drug interactions represents a major safety concern, especially when the affected (object) drug has a narrow therapeutic index (ZHOU *et al.,* 2007; BUTTERWECK and DERENDORF, 2008), it is absolutely pertinent to investigate the potential for pharmacokinetic-based interactions between *C. papaya* extract and amiodarone (AM; a narrow therapeutic index drug).

Actually, despite its narrow therapeutic index, AM [2-*n*-butyl-3-(3,5-diiodo-4-diethylaminoethoxy-benzoyl)-benzofuran; (Figure VII.1)] is one of the most widely prescribed antiarrhythmic agents (PAPIRIS *et al.,* 2010). Furthermore, the pharmacokinetics of AM and of its main metabolite, mono-*N*-desethylamiodarone (MDEA; Figure VII.1), are complex and have shown large inter-individual variability (OHYAMA *et al.,* 2000; VAN HERENDAEL and DORIAN, 2010). In fact, AM has a variable oral bioavailability and undergoes extensive enterohepatic recirculation after intestinal absorption (VAN HERENDAEL and DORIAN, 2010); moreover, the parent drug and its main metabolite are highly lipophilic and tend to accumulate extensively in several tissues (WOLKOVE and BALTZAN, 2009). On the other hand, AM has been associated with a variety of life-threatening adverse events, including thyroid dysfunction, pulmonary toxicity and hepatic toxicity (KAHALY *et al.,* 2007; WOLKOVE and

BALTZAN, 2009; LAHBABI *et al.,* 2012), and the drug has also been implicated in relevant clinical drug interactions (SIDDOWAY, 2003; EDWIN, JENNINGS and KALUS, 2010; KARIMI *et al.,* 2010; ROUGHEAD *et al.,* 2010). Thus, taking into account all the reasons previously referred, this work was planned in order to investigate whether a standardized extract of the fruit of *C. papaya* influence the rate and extent of exposure to AM in rats, following their simultaneous oral co-administration and after a pre-treatment period for 14-day with *C. papaya* extract.

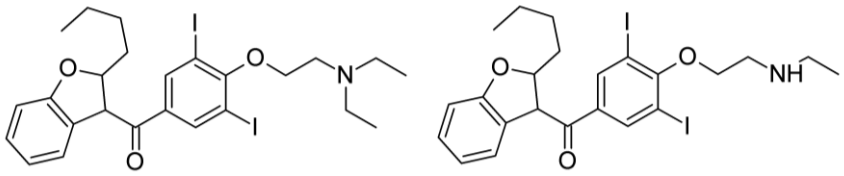

Figure VII.1. – Chemical structures of amiodarone (AM) and its major metabolite mono-*N*-desethylamiodarone (MDEA).

VII.3. MATERIALS AND METHODS

VII.3.1 DRUGS AND MATERIALS

C. papaya extract with a proteolytic activity higher than 6000 NFPU/mg obtained from papaya fruit was purchased from Bio Serae Laboratories (Bram, France). The certificate of analysis number 420015 - batch 0810960 is provided as *Supplementary data*. Carboxymethylcellulose sodium salt for preparation of extract suspension was obtained from Sigma (St. Louis, MO, USA). A commercial formulation (ampoules) of AM hydrochloride 50 mg/mL solution for intravenous injection was used for oral administration to rats after appropriate dilution with 5% glucose intravenous solution for infusion (B. Braun Medical, Portugal). Other compounds used were sodium chloride 0.9% solution for injection (Labesfal, Portugal); heparin sodium 5000 U.I./mL for injection (B. Braun Medical, Portugal); ketamine for injection (Imalgene 1000) and xylazine for injection (Vetaxilaze 20). Introcan® Certo IV indwelling cannula (22G; 0.9 × 2.5 mm) made of polyurethane from B. Braun Melsungen AG (Melsungen, Germany).

VII.3.2 ANIMALS

Adult male Wistar rats (355 ± 28 g) of approximately 10 weeks old were obtained from local animal facilities (Faculty of Health Sciences of the University of Beira Interior, Covilhã, Portugal). The rats were maintained under controlled environmental conditions (temperature 20±2 °C; relative humidity 55±5%; 12-h light/dark cycle). The animals were allowed free access to a standard rodent diet (4RF21, Mucedola, Italy) during almost all experimental procedures and tap water was available *ad libitum*. At the night of the day before AM administration, a lateral tail vein of each rat was cannulated, under anaesthesia [ketamine (90 mg/kg)/xylazine (10 mg/kg); i.p. injection], by insertion of an Introcan® Certo IV indwelling cannula (22G; 0.9 × 2.5 mm) used for serial blood sampling. The rats fully recovered from anaesthesia overnight and were fasted for 12-14 h before AM administration and maintained with free access to water. In order to avoid the effect of the food on the oral bioavailability of AM an additional fasting period was considered (4 h post-dose). Oral treatments of the rats with *C. papaya* extract and AM were performed by gavage. Blood sampling was conducted in conscious and freely moving rats appropriately restrained only at

the moment of blood collection; the exception was at the last blood sampling that was taken by a terminal procedure (decapitation and exsanguination under anaesthesia). All the animal experiments were conducted in accordance with the European Directive (2010/63/EU) for the accommodation and care of laboratory animals and the experimental procedures were reviewed and approved by the Portuguese Veterinary General Division.

VII.3.3 EXPERIMENTAL DESIGN AND PHARMACOKINETIC STUDIES

A simultaneous oral co-administration study with a single-dose of *C. papaya* extract and AM, and a 14-day repeated oral pre-treatment study with *C. papaya* extract followed by an oral dose of AM on the 15th day were performed to investigate the effects of *C. papaya* fruit extract on the pharmacokinetics of AM. The dose established for AM was 50 mg/kg since it provides plasma concentrations in rats within the drug therapeutic range (0.5-2 μg/mL) (SHAYEGANPOUR, JUN and BROCKS, 2005; SHAYEGANPOUR, HAMDY and BROCKS, 2008). On the other hand, the dose of *C. papaya* was selected based on the average dose recommended to humans by the supplier of the extract (Bio Serae Laboratories) and taking into account the Food and Drug Administration (FDA) Guidance for Industry on conversion of animal doses to human equivalent doses based on body surface area (GUIDANCE FOR INDUSTRY, 2005); additionally, a 10-fold potentiating interaction factor was considered in order to avoid potential false-negative results for herb-drug interaction associated to interspecies differences. In each day of the experiments *C. papaya* extract was suspended in 0.5% carboxymethylcellulose aqueous solution affording a suspension of herbal extract at 123 mg/mL. AM commercial injectable solution (50 mg/mL) was also appropriately diluted with 5% glucose solution to extemporaneously prepare an AM solution at 12.5 mg/mL. Appropriate volumes of *C. papaya* extract suspension (10 mL/kg of body weight) and of AM solution (4 mL/kg of body weight) were orally administered to rats by gavage.

In the first pharmacokinetic study, twelve Wistar rats were randomly divided into two groups (experimental and control groups). Rats of the experimental group (n = 6) were concomitantly treated with a single-dose of *C. papaya* extract (1230 mg/kg, p.o.) and a single-dose of AM (50 mg/kg, p.o.); the extract suspension was administered immediately before AM. Rats of the control group (n = 6) received, instead of the *C. papaya* extract suspension, the corresponding volume of 0.5% carboxymethylcellulose aqueous solution (extract vehicle).

In the second pharmacokinetic study, twelve Wistar rats were also randomly divided into two groups. Rats assigned to the experimental group (n = 6) were orally pre-treated with *C. papaya* extract (1230 mg/kg, p.o.) once daily for 14 consecutive days (short-term repeated dose pre-treatment). Rats allocated to the control group (n = 6) were administered with an equivalent volume of vehicle for the same period of time. During the pre-treatment period, the rats were kept in 12-h light/dark cycle animal room with controlled temperature and humidity, as indicated in *section VII.3.2*, and the free access to a standard rodent diet and tap water was allowed. On the 15th day, the rats of both groups (experimental and control) were gavaged with the single-dose of AM (50 mg/kg, p.o.).

In both pharmacokinetic studies the treatments with *C. papaya* extract (or vehicle) and/or AM were always carried out on the morning between 9:00 am and 11:45 am. At the night of the day before AM administration, the rats were anaesthetized for cannulation of a lateral tail vein and were fasted overnight as described above *(section VII.3.2).* On the day after, multiple serial blood samples (approximately 0.3 mL per sample) were collected through the cannula into heparinized tubes before dosing and at 0.25, 0.5, 1, 2, 4, 6, 8 and 12 h following AM administration; then, at 24 h post-dose, blood and tissues (heart, liver, kidneys and lungs) were also harvested after decapitation of the rats. The blood samples were centrifuged at 4000 rpm for 10 min (4 °C) to separate the plasma which was stored at –20 °C until analysis. After exsanguinations, liver, kidneys, heart and lungs were excised and stored at –20 °C; the organs were weighed and homogenized in distilled water (3 mL of water per gram of tissue) before analysis of tissue homogenates samples.

VII.3.4 ANALYSIS OF AMIODARONE AND MONO-*N*-DESETHYLAMIODARONE

Plasma and tissue concentrations of AM and its main metabolite MDEA were determined by using a liquid-liquid extraction procedure followed by high-performance liquid chromatography-diode array detection assay previously developed and validated (RODRIGUES *et al.,* 2013a).

VII.3.5 PHARMACOKINETIC ANALYSIS

The plasma concentration *versus* time data for AM and MDEA obtained from each individual rat were submitted to a non-compartmental pharmacokinetic analysis using the WinNonlin® version 4.1 (Pharsight Co, Mountain View, CA, USA). The peak plasma concentrations (C_{max}) of AM and MDEA and the time to reach C_{max} (t_{max}) were obtained directly from the experimental data. Other pharmacokinetic parameters estimated from the individual plasma concentration-time profiles included: area under the concentration-time curve (AUC) from time zero to the last sampling time at which concentrations were at or above the limit of quantification (LOQ; 0.100 µg/mL) of the method (AUC_{0-t}), calculated by the linear trapezoidal rule; AUC from time zero to infinite ($AUC_{0-\infty}$), calculated from AUC_{0-t} + (C_{last}/k_{el}), where C_{last} is the last quantifiable concentration and k_{el} is the apparent terminal elimination rate constant calculated by log-linear regression of the terminal segment of the concentration-time profile; apparent terminal elimination half-life ($t_{1/2el}$) and mean residence time (MRT). The concentrations lower than the LOQ of the assay were taken as zero for all calculations.

VII.3.6 SHORT-TERM REPEATED DOSE EFFECT OF *CARICA PAPAYA* EXTRACT ON BODY WEIGHT

In the short-term *C. papaya* repeated dose study the body weight of the rats treated with *C. papaya* extract (1230 mg/kg/day, p.o.; experimental group) or vehicle (control group) were adequately registered on the first day and also on the last day (14^{th}) of these treatments in order to investigate the effect of the herbal extract on body weight changes.

VII.3.7 STATISTICAL ANALYSIS

Data were reported as the mean ± standard error of the mean (SEM). Comparisons between two groups were usually performed using unpaired two-tailed Student's *t*-test; for body weight comparisons within the same group the paired Student's *t*-test was applied. The differences were considered to be statistically significant for a *p*-value lower than 0.05 ($p < 0.05$).

VII.4. RESULTS

VII.4.1 SIMULTANEOUS CO-ADMINISTRATION OF *CARICA PAPAYA* AND AMIODARONE

The mean plasma concentration-time profiles (n = 6) of AM and its main metabolite (MDEA) obtained after the co-administration of rats with a single-dose of *C. papaya* extract (1230 mg/kg, p.o.) or vehicle (control group) and a single-dose of AM (50 mg/kg, p.o.) are shown in Figure VII.2.

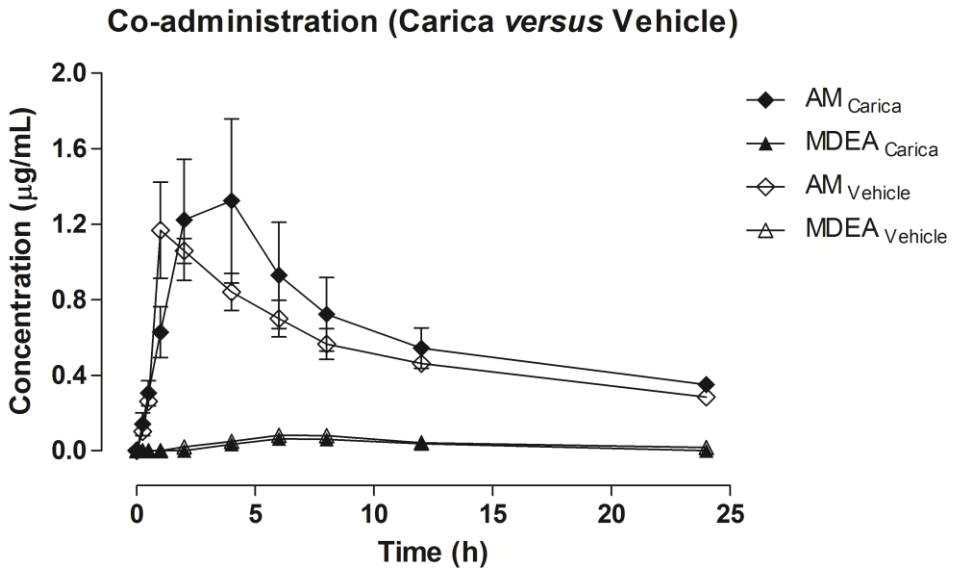

Figure VII.2 – Mean plasma concentration-time profiles of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) obtained, over a period of 24 h, from rats simultaneously treated with a single-dose of *Carica papaya* extract (1230 mg/kg, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and AM (50 mg/kg, p.o.) by oral gavage. Symbols represent the mean values ± standard error of the mean (SEM) of six determinations per time point (n = 6).

Up to 24 h post-dosing no statistically significant differences (p > 0.05) were found between both groups of rats (*C. papaya versus* vehicle) regarding the mean plasma concentrations of AM achieved at each sampling time points. The plasma concentrations of MDEA were similar in both groups, showing concentration values near or below the LOQ (0.100 µg/mL) of the analytical method, which were manifestly lower than those obtained for AM. The main pharmacokinetic parameters estimated for AM and MDEA after a non-

compartmental analysis of their individual plasma concentration-time profiles are summarized in Table VII.1.

Table VII.1 – Pharmacokinetic parameters estimated by non-compartmental analysis of the plasma concentration-time profiles of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA, major metabolite of AM) obtained in rats after the simultaneous co-administration in single-dose of *Carica papaya* extract (1230 mg/kg, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), with AM (50 mg/kg, p.o.) by oral gavage (*n* = 6, unless otherwise noted).

Parameter	AM_{Carica}		$AM_{Vehicle}$	
	AM	MDEA	AM	MDEA
t_{max} (h)	2.50 ± 0.50	6.00 ± 0.00[a]	1.83 ± 0.48	7.20 ± 1.36[b]
C_{max} (µg/mL)	1.530 ± 0.409	0.127 ± 0.020[a]	1.378 ± 0.179	0.125 ± 0.012[b]
AUC_{0-t} (µg.h/mL)	15.588 ± 3.414	ND	12.774 ± 0.688	ND
$AUC_{0-\infty}$ (µg.h/mL)	25.693 ± 3.488	ND	21.431 ± 2.077	ND
k_{el} (1/h)	0.0470 ± 0.0102	ND	0.0433 ± 0.0082	ND
$t_{1/2el}$ (h)	19.87 ± 5.32	ND	20.73 ± 5.74	ND
MRT (h)	29.79 ± 8.04	ND	28.64 ± 7.74	ND

ND, not determined.
[a]n = 3; [b]n = 5.

Considering the paucity of quantifiable plasma concentrations obtained for MDEA, it was only possible to present the C_{max} and t_{max} parameters (Table VII.1). Overall, the C_{max} of AM was attained later in the group treated with *C. papaya* comparatively to the vehicle (control) group. No statistically significant differences in the mean pharmacokinetic parameters were found in terms of magnitude and extent of systemic exposure to AM and its main metabolite (MDEA) among both groups (Table VII.1). Taking into consideration the information derived from Figure VII.3, it also appears that following the simultaneous co-administration of *C. papaya* and AM the magnitude of systemic exposure (as assessed by C_{max}) and the extent of systemic exposure to AM (as assessed by AUC_{0-t} and $AUC_{0-\infty}$) are similar among experimental and control groups (ratios near to unity).

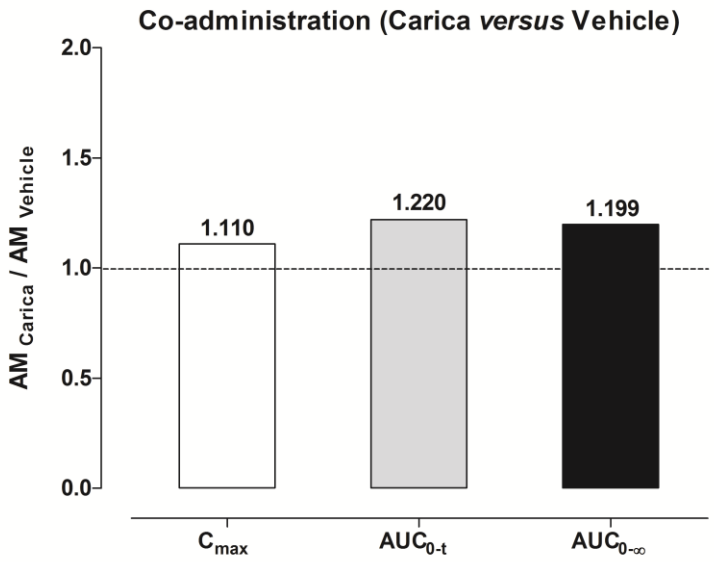

Figure VII.3. – Ratios for the main plasma pharmacokinetic parameters (C_{max}, AUC_{0-t} and $AUC_{0-\infty}$) estimated for amiodarone (AM) in rats simultaneously treated in single-dose with *Carica papaya* extract (1230 mg/kg, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and AM (50 mg/kg, p.o.) by oral gavage.

To evaluate the distribution of AM and MDEA in the presence and absence of the co-administration with the *C. papaya* extract, the animals were sacrificed at 24 h post-dosing and several tissues were excised and analysed. The mean concentrations of AM and MDEA in heart, lung, liver and kidney tissues, and also in plasma at the same time point (24 h post-dose) are shown in Figure VII.4.

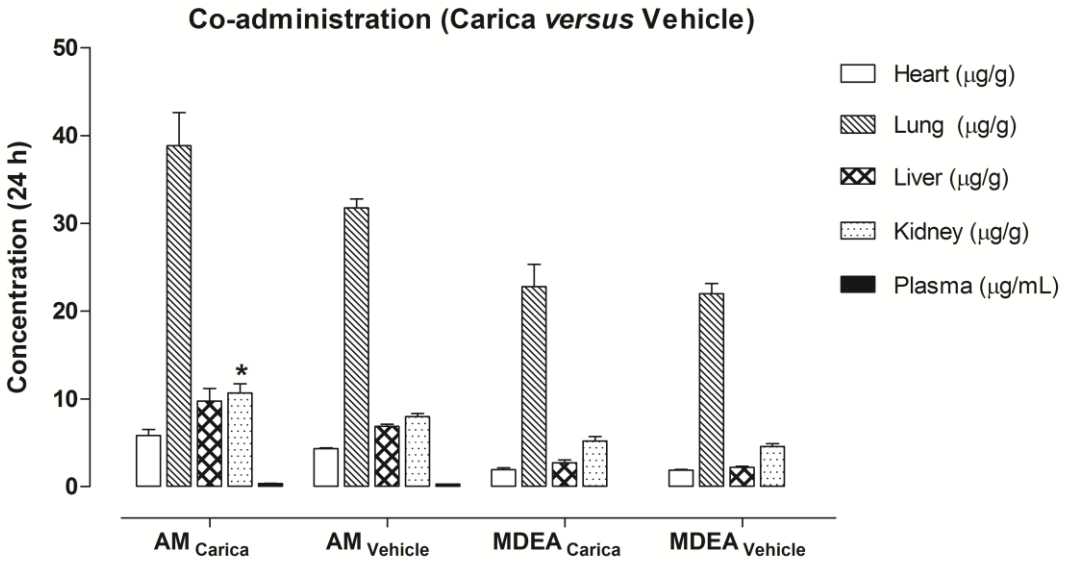

Figure VII.4 – Mean plasma and tissue (heart, lung, liver and kidney) concentrations of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) obtained, at 24 h post-dose, from rats simultaneously treated with a single-dose of *Carica papaya* extract (1230 mg/kg, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and AM (50 mg/kg, p.o.) by oral gavage. Data are expressed as the mean values ± standard error of the mean (SEM) of six determinations (n = 6). *p < 0.05 compared to control (vehicle).

The tissue concentrations of AM and MDEA were markedly higher than those determined in plasma. However, taking into account the comparisons performed between the experimental (*C. papaya*) and the control (vehicle) groups, statistically significant differences were only detected for AM concentrations in kidney tissue (p < 0.05), being significantly higher in the kidney tissue of rats treated with the herbal extract.

VII.4.2 SHORT-TERM REPEATED DOSE PRE-TREATMENT WITH *CARICA PAPAYA* FOLLOWED BY ADMINISTRATION OF AMIODARONE

The rats were administered for 14 days with *C. papaya* extract (1230 mg/kg, p.o.) or vehicle (control group) in order to investigate the influence of *a* short-term repeated dose pre-treatment with *C. papaya* extract on the pharmacokinetics of AM, which was only administered on the 15th day as a single-dose of 50 mg/kg (p.o.). The mean plasma concentration-time profiles (n = 6) for AM and its main metabolite (MDEA) are depicted in Figure VII.5.

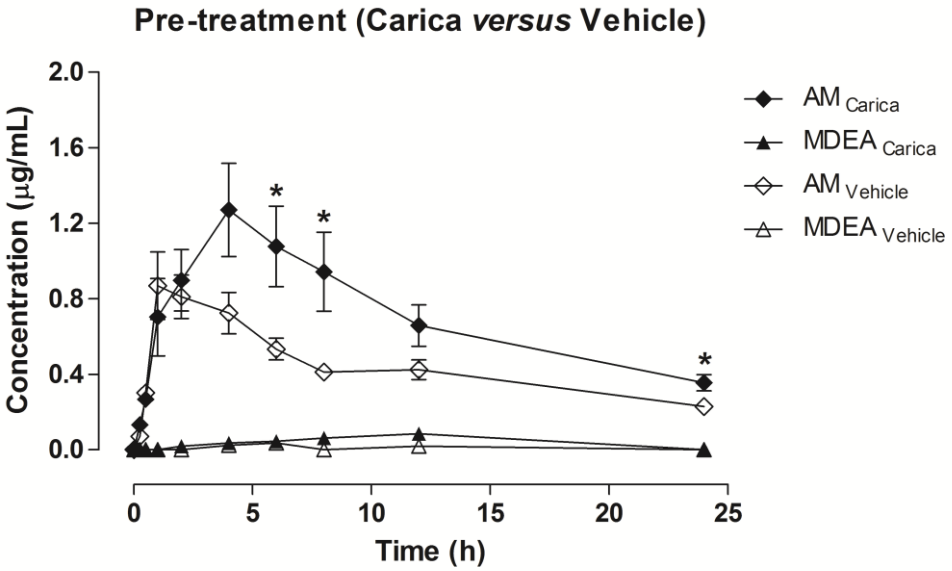

Figure VII.5 – Mean plasma concentration-time profiles of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) obtained, over a period of 24 h, from rats submitted to a 14-day pre-treatment period with *Carica papaya* extract (1230 mg/kg/day, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and treated on the 15th day with a single-dose of AM (50 mg/kg, p.o.) by oral gavage (n = 6). Symbols represent the mean values ± standard error of the mean (SEM) of six determinations per time point (n = 6). *p < 0.05 compared to control (vehicle).

Comparing the mean plasma concentrations for AM in both groups, there are statistically significant differences at 6, 8 and 24 h post-dosing (p < 0.05) which were higher in the group pre-treated with *C. papaya* extract. The corresponding pharmacokinetic parameters are listed in Table VII.2.

Table VII.2 – Pharmacokinetic parameters estimated by non-compartmental analysis of the plasma concentration-time profiles of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA, major metabolite of AM) obtained in rats submitted to a 14-day pre-treatment period with *Carica papaya* extract (1230 mg/kg/day, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and treated on the 15th day with a single-dose of AM (50 mg/kg, p.o.) by oral gavage (*n* = 6, unless otherwise noted).

Parameter	AM_{Carica}		$AM_{Vehicle}$	
	AM	MDEA	AM	MDEA
t_{max} (h)	3.17 ± 0.54	10.50 ± 2.12[a]	2.17 ± 0.60	7.33 ± 2.40[b]
C_{max} (µg/mL)	1.436 ± 0.224	0.135 ± 0.011[a]	0.952 ± 0.157	0.119 ± 0.014[b]
AUC_{0-t} (µg.h/mL)	16.932 ± 2.560*	ND	10.532 ± 0.889	ND
$AUC_{0-\infty}$ (µg.h/mL)	26.635 ± 2.527[#]	ND	15.325 ± 0.949	ND
k_{el} (1/h)	0.0541 ± 0.0127	ND	0.0533 ± 0.0082	ND
$t_{1/2el}$ (h)	18.30 ± 5.07	ND	14.36 ± 1.85	ND
MRT (h)	27.05 ± 7.07	ND	20.97 ± 2.42	ND

ND, not determined.
[a]n = 4; [b]n = 3.
*$p < 0.05$, significantly different from the control group.
[#]$p < 0.005$, significantly different from the control group.

Regarding the data shown in Figure VII.6, it was evident an increase (50.8%) in the mean peak plasma concentration (C_{max}) of AM, but this did not reach statistical significance, as well as an increase of approximately 60.8-73.8% in the extent of systemic drug exposure (as assessed by AUC_{0-t} and $AUC_{0-\infty}$) in the rats pre-treated with *C. papaya* extract.

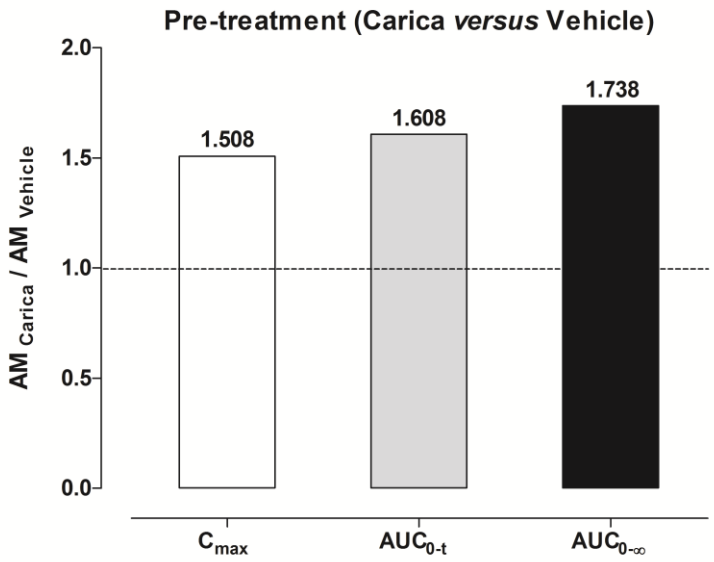

Figure VII.6. – Ratios for the main plasma pharmacokinetic parameters (C_{max}, AUC_{0-t} and $AUC_{0-\infty}$) estimated for amiodarone (AM) in rats submitted to a 14-day pre-treatment period with *Carica papaya* extract (1230 mg/kg/day, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and treated on the 15th day with a single-dose of AM (50 mg/kg, p.o.) by oral gavage.

Indeed, the *C. papaya* extract pre-treatment of the rats only determined a statistically significant increase in the extent of systemic exposure to AM (as assessed by AUC_{0-t} and $AUC_{0-\infty}$). In the experimental (*C. papaya*) group, it was also observed a considerable delay in reaching C_{max} of AM. The plasma concentrations of MDEA were near or below the LOQ (0.100 µg/mL) of the method in both groups of rats.

To assess the impact of a 14-day pre-treatment period with *C. papaya* extract (experimental group) on the distribution and metabolism of AM in rats, the concentrations of AM and its major metabolite (MDEA) were also determined in various tissues (additionally to plasma) at 24 h post-dose and the data are shown in Figure VII.7.

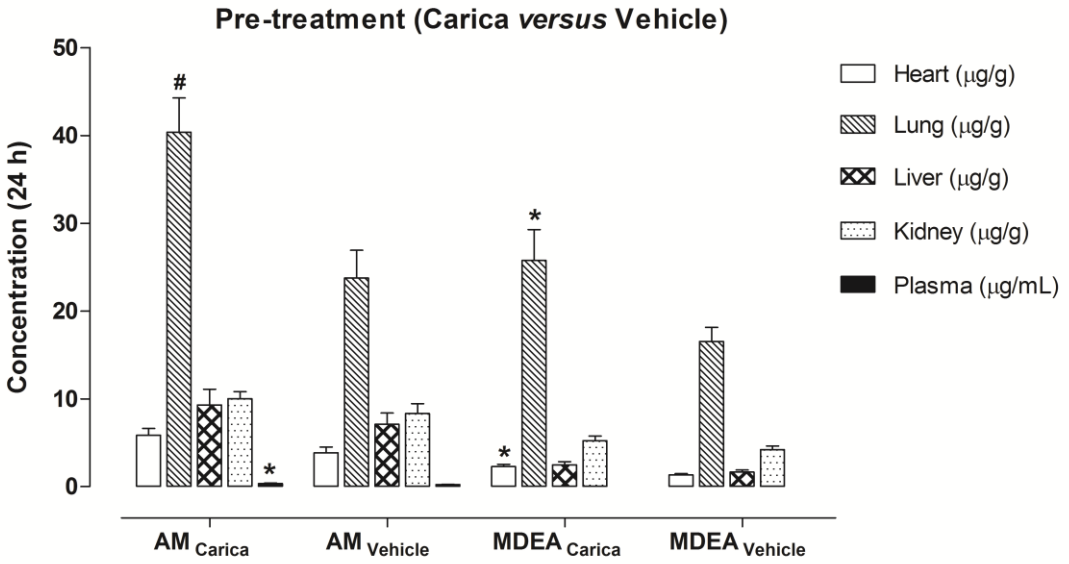

Figure VII.7 – Mean plasma and tissue (heart, lung, liver and kidney) concentrations of amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) obtained, at 24 h post-dose, from rats submitted to a 14-day pre-treatment period with *Carica papaya* extract (1230 mg/kg/day, p.o.), or vehicle (0.5% carboxymethylcellulose aqueous solution), and treated on the 15th day with a single-dose of AM (50 mg/kg, p.o.) by oral gavage. Data are expressed as the mean values ± standard error of the mean (SEM) of six determinations (n = 6). $^{*}p < 0.05$ and $^{\#}p < 0.01$ compared to control (vehicle).

Once again, the concentrations of both compounds (AM and MDEA) in the tissues were distinctly greater than those measured in plasma. However, despite the statistically significant differences detected in the plasma concentrations of AM at 6, 8 and 24 h post-dose between both groups (Figure VII.5), statistically significant differences in the mean tissue concentrations of AM were only found in the lung tissue, while MDEA concentrations obtained from rats pre-treated with *C. papaya* extract were higher in lung and heart tissue.

VII.4.3 SHORT-TERM REPEATED DOSE EFFECT OF *CARICA PAPAYA EXTRACT* ON BODY WEIGHT

The changes in body weight of the rats submitted to a 14-day treatment period with *C. papaya* extract (1230 mg/kg/day, p.o) or vehicle are pointed out in Figure VII.8.

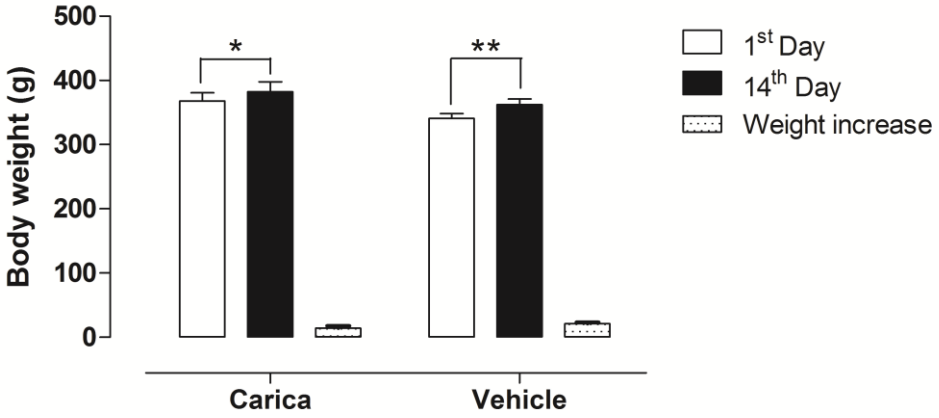

Figure VII.8 – Effects on the body weight of the rats induced by the short-term repeated dose treatment (14-day period) with *Carica papaya* extract (1230 mg/kg/day, p.o.) and vehicle (0.5% carboxymethylcellulose aqueous solution) by oral gavage. *p < 0.01 and **p < 0.001, 1st day *versus* 14th day.

Between the 1st and the 14th day, statistically significant increases in the body weight of rats treated with *C. papaya* extract (p < 0.01) or vehicle (p < 0.001) were observed. However, the magnitude of the increase in their body weight was similar in both groups (*C. papaya versus* vehicle).

VII.5. DISCUSSION

Most of the herb-drug interaction studies found in literature have been conducted *in vitro* employing concentrations usually higher than those clinically relevant ones (VENKATARAMANAN, KOMOROSKI and STROM, 2006) and, up to date, few significant drug interactions have actually been accurately predicted from *in vitro* assays (MARKOWITZ, VON MOLTKE and DONOVAN, 2008). Thus, taking into account these limitations, the present work evaluated the potential of interaction between *C. papaya* extract and AM in a whole animal model, the Wistar rat.

Overall, our results showed that the single-dose co-administration of *C. papaya* extract and AM caused an apparent delay in the t_{max}, but did not alter the extent of systemic exposure to AM (as assessed by AUC_{0-t} and $AUC_{0-\infty}$). This apparent delay in t_{max} is not expected to change the efficacy of AM and it is unlikely to be clinically important. At this time point it is interesting to be emphasised that after the co-administration of AM with other herbal extracts claimed to be useful in weight loss/weight management (e.g. *Citrus aurantium* extract, *Fucus vesiculosus* extract and *Paullinia cupana* extract) a delay in the time to reach C_{max} was also observed; however, the co-administration with *F. vesiculosus* extract or *P. cupana* extract significantly decreased the systemic exposure to AM in the rats treated with the herbal extracts (RODRIGUES *et al.,* 2012; RODRIGUES, ALVES and FALCÃO, 2013; RODRIGUES *et al.,* 2013b).

Moreover, because of the central role that the induction of cytochrome P450 (CYP) isoenzymes and P-glycoprotein (P-gp) plays on drug-drug and herb-drug interactions, and bearing in mind that the induction mechanisms are time-dependent, the interference of *C. papaya* extract on the pharmacokinetics of AM was also evaluated by administering the herbal extract for 14 consecutive days until 24 h before administering a single-dose of drug. In the rats pre-treated with *C. papaya* extract during the 14 days, a statistically significant increase in the extent of systemic drug exposure was observed comparatively with control group (as assessed by AUC_{0-t} and $AUC_{0-\infty}$). However, no significant differences were found in the peak of systemic exposure to AM (as assessed by C_{max}). Thus, to explain the greater systemic exposure to AM in the experimental (*C. papaya*) group we hypothesize that the herbal extract or some of its phytochemical constituents could have antimotility properties determining the inhibition of the intestinal propulsion movements (EZIKE *et al.,* 2009). The reduction of gastrointestinal motility can prolong the transit time of drugs and, consequently,

it can increase the extent of the absorption of slightly soluble drugs, such as AM (TARIRAI, VILJOEN and HAMMAN, 2010). In fact, the rate-limiting step of AM intestinal absorption is its low aqueous solubility, given that it occurs by passive diffusion (a non-saturable transport process) (MARTÍN-ALGARRA *et al.,* 1994, 1997).

Another possibility to explain the higher AUC_{0-t} and $AUC_{0-\infty}$ values for AM in the group of rats pre-treated with *Carica papaya* extract could be related to a time-dependent inhibitory effect on CYP isoenzymes and/or P-gp-mediated efflux activity. Theoretically, a drug that is a dual substrate for CYPs and P-gp has much higher potential for drug interactions with herbs that also modulate CYP3A4 and P-gp (ZHOU *et al.,* 2007). In fact, AM is metabolized by several CYP isoenzymes including CYP1A1/2, CYP2C8, CYP2C19, CYP2D6 and CYP3A4 (OHYAMA *et al.,* 2000; ELSHERBINY, EL-KADI and BROCKS, 2010) and is also a substrate of P-gp (SHAPIRO and SHEAR, 2002; KALITSKY-SZIRTES *et al.,* 2004). In addition, there are some evidences of effects of *C. papaya* on CYPs and/or P-gp activity; the fruit of *C. papaya* seems to produce inhibitory effects on CYP3A activity and CYP2E1 activity in human and mouse liver microsomes respectively (HIDAKA *et al.,* 2004; CHATUPHONPRASERT and JARUKAMJORN, 2012). The juice of *C. papaya* has also shown a weak inhibitory effect on the CYP2C9 activity in human liver microsomes (HIDAKA *et al.,* 2008). OGA *et al.* (2012) also reported the inhibition of P-gp in Caco-2 cells mediated by the leaf extract of *C. papaya*. In addition, the aqueous extract of *C. papaya* leaves significantly increased (54.5%) the apparent permeability of the P-gp substrate digoxin in the mucosal-to-serosal direction in intestinal segments mounted in Ussing chambers (OGA, SEKINE and HORIE, 2013). However, the co-administration of the leaf extract of *C. papaya* and digoxin to rats did not determine a significant increase in the extent of systemic drug exposure (OGA, SEKINE and HORIE, 2013).

Based on the herb-drug interaction data obtained in the present work between *C. papaya* extract and AM, it is suggested that the herbal extract has impact on the pharmacokinetics of AM, particularly after the repeated treatment with the *C. papaya* extract; indeed, the 14-day pre-treatment period with *C. papaya* induced an increase in the bioavailability of AM. Even so, it should be taken in account that results from animal experiments cannot be directly extrapolated to humans; however, bearing in mind the studies of MENG *et al.* (2001) and SHAYEGANPOUR, JUN and BROCKS (2005), the rat appears to be an appropriate model for man in this case. Nevertheless, to reliably assess the safety of administration of *C. papaya* extract and AM specific clinical trials should be performed.

Additionally, in the present study, the increase of body weight of the rats pre-treated during 14 days with *C. papaya* extract or vehicle was comparable. Despite there are evidence in literature that *C. papaya* have anti-obesity effects, our data do not support these findings. In a study conducted by ATHESH *et al.* (2012) in which rats fed with high fat diet were treated during a 14-day period with *C. papaya* extract at doses in the range of 200-600 mg/kg only a slight reduction in body weight gain was observed. Indeed, the anti-obesity effect was more evident after 45 days of treatment with *C. papaya* fruit extract. In other studies the treatment with *C. papaya* seed extract induced a reduction in body weight gain of rats when doses of 100 to 400 mg/kg were administered over 30 days and doses of 2000 mg/kg were administered during 14 days (ADENEYE and OLAGUNJU, 2009); moreover, a 28-day treatment period with 5 to 20% of ground bark extract of *C. papaya* in the diet also determined a reduction in body weight gain of rats comparatively to the control group (DURU *et al.,* 2012). At last, in a study conducted by GOYAL *et al.* (2010) no significant changes were observed in the body weight of the rats treated during 52 days with doses of 50 to 500 mg/kg of the methanol sub-fraction of *C. papaya* seeds .

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VII.7. SUPPLEMENTARY DATA

Certificate of analysis of *Carica papaya* extract provided by Bio Serae Laboratories (Ref. 420015 – Batch 0810960).

Bio serae
Laboratoires S.A.S.

PAPAYA EXTRACT

Carica papaya

CERTIFICATE OF ANALYSIS
Ref. 420015 - Batch 0810960

	Methods	Specifications	Results
MAIN CHARACTERISTICS			
Appearance	Bio Serae	fine powder	complies
Color	Bio Serae	white to yellowish	complies
Nature	Bio Serae	standard. latex purified from papaye	complies
Odor	Bio Serae	caracteristic	complies
Gamma ray treatment	-	Not treated	complies
Taste	Bio Serae	caracteristic	complies
Carrier	Bio Serae	maltodextrin	complies
ACTIVE INGREDIENTS			
Proteolytic activity (NFPU/mg)	spectrophotometric	≥ 6 000	7 500
MICROBIOLOGICAL			
Enterobacteria (CFU/g)	NF ISO 21528 - 2	< 1 000	< 10
Escherichia coli (CFU/10g)	Eur. Ph. 2000 [§2.6.13]	negative	negative
Total plate count (CFU/g)	NF ISO 4833	< 10 000	250
Yeasts and moulds (CFU/g)	AOAC 997.02	< 500	< 10
Salmonella (CFU/25g)	BKR 23/04-12/07	negative	negative
Staphylococcus aureus (DNAse +) (CFU/10g)	Eur. Ph. 2000 [§2.6.13]	negative	negative
OTHER ANALYTICAL DATA			
Dry matter (%)	Eur.Ph.1997(§2.2.32 - 105°C)	> 90	98,3
CONTAMINANTS			
Heavy metals (lead eq.) (ppm)	Eur. Ph. 2000 [§2,4,6D]	< 10	< 10
GMO status	1829-1830/2003CE	Conventional ingredient	complies

STORAGE

24 months if kept in a dry and cool place in original intact packing

Manufacturing / analysis date : 11 / 2008
Best before : 11 / 2010

The information here above is based on our current knowledge. BIO SERAE cannot be hold responsible besides the guarantees written on its supply contracts, based on the fact that it does not control the final use of this product. It is the buyer's responsibility to comply with local texts and laws regulating its activity and the use of this product.

CFU = Colony-Forming Unit
DM = Dry Matter
RM = Raw Matter

Bram, 25-nov-08

Qualified batch

Mr. Murat
Quality Control Department

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CHAPTER VIII

GENERAL DISCUSSION

VIII. GENERAL DISCUSSION

VIII.1. DISCUSSION

Although each of the experimental chapters covering the research work performed has its own discussion (from Chapter II to VII), this section is intended to discuss in a more integrated and broader manner all the research studies presented in this thesis. The work underlying the present thesis aimed at the general objectives proposed at the beginning of this dissertation.

In recent years, the increasing interdependence between countries gave rise to the need of international acceptance of the results from the analytical methods and, consequently, to ensure a common minimum level of quality the use of validated analytical methods is becoming an indispensable condition. Actually, since a long time that the results generated by non-validated methods are devoid of any meaning and are not recognized by the authorities (HARTMANN *et al.,* 1998).

In fact, the analytical methods for the quantification of drugs and metabolites in biological samples play a decisive role, by the quality of the data provided, in the evaluation and interpretation of results arising from preclinical, clinical and biopharmaceutical studies (SHAH *et al.,* 2000; GUIDANCE FOR INDUSTRY, 2001). In this regard, the role of bioanalytical sciences is of the utmost importance in development programs of new drugs and novel formulations and even in the implementation of therapy. Analytical data inaccurate or unreliable can be the cause of a wrong diagnosis or treatment (BUICK *et al.,* 1990; CHANDRAN and SINGH, 2007). To counteract these undesirable issues and to obtain reliable results which can be satisfactorily interpreted, the application of an analytical technique fully validated is essential (SHAH *et al.,* 2000).

Thus, before a bioanalytical method can be implemented for routine use it must be validated in order to demonstrate its reliability for the determination of an analyte of interest in a specific biological matrix. The validation of an analytical method is the systematic process that aims to demonstrate that the methodology used is acceptable for the required application and ensures a better confidence of the data obtained (SHAH *et al.,* 2000; GUIDANCE FOR INDUSTRY, 2001; CHANDRAN and SINGH, 2007). Nowadays the validation of bioanalytical methods to quantify small molecules in biological samples is based on the

conference report published by SHAH *et al.* (2000) on *Bioanalytical Method Validation – A Revisit with a Decade of Progress*, on the present *Guidance for Industry – Bioanalytical Method Validation* (GUIDANCE FOR INDUSTRY, 2001) and on the recent *Guideline on bioanalytical method validation* (EUROPEAN MEDICINES AGENCY, 2011a). Accordingly, at least the following parameters should be evaluated: selectivity, calibration model (linearity), limit of quantification (LOQ), precision, accuracy, recovery and stability of compounds. The success of the validation process implies, however, the previous existence of an analytical method appropriately developed (DADGAR *et al.*, 1995). In this way, it is not possible that the process of validating an analytical method is staked out of the respective conditions of development, since the validation studies determine if the state of development of the method is quite satisfying or if alterations are required to improve the analytical procedures and subsequent revalidation (CHANDRAN and SINGH, 2007).

In the process of development and validation of bioanalytical methods also should be considered multiple variables at once meet the biological matrix used (which should be the same of specimens intended), the procedure for collecting the sample and the time that elapses until the analysis, the chromatographic separation of the analyte, the detection system and the data processing (BRESSOLE, BROMET-PETIT and AUDRAN, 1996; SHAH *et al.,* 2000). As a result, the development of a new analytical method is the most critical stage of the process and depending on the desired objectives and the experience of the investigator may be carried out in various ways. When considering the two extreme situations, the development may involve only the adaptation of an existing method, with minor changes appropriate to the new application, or may involve innovative ideas and methodologies requiring harder work and often it is uncertain whether the degree of development required will be achieved (EURACHEM GUIDE, 1998).

Taking into account these considerations about validation of bioanalytical methods and the general scope of the work to be developed, the first task considered was the development and fully validation of a HPLC method to simultaneously quantify amiodarone (AM) and mono-*N*-desethylamiodarone (MDEA) in human plasma using a sample preparation involving microextraction by packed sorbent (MEPS). In this way, it was possible to make available a novel and innovative bioanalytical tool using for the first time MEPS as sample preparation procedure. The method proved to be selective, sensitive enough, precise and accurate, and linear in a wide concentration range, making it an important analytical tool for

supporting the therapeutic drug monitoring of AM in clinical practice and also to support pharmacokinetic-based studies, such as bioequivalence and bioavailability studies. The MEPS procedure developed presents several advantages in comparison with conventional sample preparation techniques frequently used in bioanalysis. In addition, this technique was successfully applied to real plasma samples of highly polymedicated patients receiving AM treatment.

Hence, with the knowledge acquired during the optimization and validation of the previous technique in human plasma, a new HPLC-DAD method was then faster developed and fully validated for the quantitative determination of AM and MDEA in rat matrices (plasma and heart, liver, kidney and lung tissue homogenates). The development of a technique in human plasma enable to acquire some experience in the bioanalytical field, enabling in this way to reduce the number of animals to the minimum necessary, following the 3Rs principle, in the development of the technique in rat matrices (BALANI *et al.,* 2008; FENWICK, GRIFFIN and GAUTHIER, 2009). The method developed presents several improvements over the assays previously published for the quantification of AM and MDEA in rat plasma and tissue samples. Between them, this bioanalytical method enables the fast chromatographic analysis of AM and MDEA using simple instrumentation and uncomplicated chromatographic conditions. The sample preparation procedures employed are relatively similar to previous published methods but lower volumes of organic solvents for the extraction and lower volumes of sample were used. This aspect is fundamental when serial blood sample collection from small laboratory animals (usually rodents) is required in the course of non-terminal pharmacokinetic studies. Indeed, the experimental design planned for the following *in vivo* studies to be conducted in rats involved the harvesting of multiple blood samples from each animal.

After the development and validation of the HPLC techniques for the determination of AM and its main metabolite (MDEA) the following steps of the present thesis consisted in the evaluation of pharmacokinetic herb-drug interactions between the antiarrhythmic drug, AM, and four standardized herbal extracts used in the management of obesity (*Fucus vesiculosus*, *Paullinia cupana*, *Citrus aurantium* and *Carica papaya*). These herbal extracts were selected because they are frequently used in commercial dietary/medicinal products and were relatively easy to acquire commercially; in addition, these herbal extracts are

standardized using different bioactive phytochemicals which are probably responsible for the properties and pharmacological actions of the extracts on the body weight loss.

The extracts of *F. vesiculosus*, *P. cupana*, *C. aurantium* and *C. papaya* exist commercially under the form of tablets of the supplier Arkocapsulas® (Arkocapsulas Algas®, Arkocapsulas Guarana®, Arkocapsulas Laranja amarga® and Arkocapsulas Papaia®). All the four extracts evaluated in this thesis are present simultaneously in the multicomponent commercial product EasySlim Depur Max®. These extracts are also present in several other commercially available products; for example, *F. vesiculosus* extract is present in Celulase Gold Pearls®; *P. cupana* extract is present in Depuralina Raspberry Power®, Depuralina Ampola® and Depuralina Concentrado Sport®, Slimexcell Refirme®, Turboslim Men 24® and Drena Slim®; the combination of *P. cupana* and *C. aurantium* extracts is present in Drenafast Intense Solução Oral Cereja® and Hot Drink Slim®; *P. cupana* and *C. papaya* extract is present in Splendid Celu®; *C. aurantium* extract is present in Depuralina Concentrado Solução® and Drena Slim Raspberry Ketone®; *C. papaya* extract is present in YMEA Silhouette®. This short list of products is very illustrative of the multiplicity of herbal preparations freely accessible in the market.

The use of complementary and alternative therapies such as herbal or natural products is widespread and has increased worldwide over the last decade despite the paucity of scientific evidence about their safety and efficacy. Consequently, unexpected and significant herb-drug interactions may occur and put individuals at risk, particularly those people who use multiple medicines for co-morbid conditions. These interactions may lead to therapeutic failure and/or toxic effects, especially for drugs characterized by a narrow therapeutic index (e.g. AM) (TACHJIAN, MARIA and JAHANGIR, 2010). Undoubtedly, the information now available on herb-drug interactions is scarce and, in many cases, inappropriate. Indeed, the information published about herb-drug interactions has derived mainly from *in vitro* studies; however, as suggested by some authors, numerous herb-drug interactions reported in literature are irrelevant and misleading due to the use of inappropriately high concentrations of the extracts or their constituents lacking, therefore, *in vivo* relevance when their bioavailability is considered (COTT, 2008; MARKOWITZ, VON MOLTKE and DONOVAN, 2008). Therefore, the *in vitro* studies are valuable in this context for evaluating multiple products and multiple components, providing mechanistic information and are also easily performed (VENKATARAMANAN, KOMOROSKI and STROM, 2006). Since the *in vitro* data on herb-drug interactions cannot usually be directly extrapolated to the *in vivo*

conditions, it is important to conduct well-designed *in vivo* non-clinical studies with enough potency for human extrapolation. The *in vivo* non-clinical studies can be conducted in small laboratory animals (e.g. mice and rats). These studies have advantages of being less expensive than human clinical studies and can often provide an initial assessment of the bioavailability either for phytochemicals in pure form or in the context of a complex extract (GURLEY, 2012). The herb-drug interaction studies can also be conducted *in vivo* in humans. Indeed, the *in vivo* clinical studies are valuable to confirm the clinical significance of herb-drug interactions identified from non-clinical assays conducted in *in vivo* animal models or in *in vitro* conditions. These studies are the only ones that are definitive. However, the clinical studies are often carried out in healthy humans and not in patients; and these studies are generally expensive and several times does not provide mechanistic information (VENKATARAMANAN, KOMOROSKI and STROM, 2006).

The work developed in this thesis was performed using adult male Wistar rat as a whole-animal model. The use of female rats was also hypothesized during the experimental design of these studies; however, only male Wistar rats were included because it has been reported that the pharmacokinetics of AM is not gender-dependent and also to avoid the potential interference of menstrual cycle hormones (possible confounding factors) (SANOFI-SYNTHELABO PFIZER CANADA INC., 2010). As previously referred, the rat was selected because this rodent species shares many of the pharmacokinetics properties of AM with humans and has been regarded as one of the best models among the small laboratory animals for studying pharmaco-toxicological aspects concerning AM; although some metabolic differences exist, it has also been demonstrated that MDEA is the principal metabolite of AM in both species (rat and human) (PLOMP *et al.,* 1987; NAJJAR, 2001; ELSHERBINY and BROCKS, 2010; ELSHERBINY, EL-KADI and BROCKS, 2010).

Globally, from the results obtained with the four herbal extracts tested we can conclude that *F. vesiculosus*, *P. cupana* and *C. papaya* extracts have more pronounced effects on the pharmacokinetics of AM. Briefly, *F. vesiculosus* extract and *P. cupana* extract following the co-administration with AM showed a significant reduction of the peak concentration of AM as well as a reduction in the extent of systemic exposure to AM. In both cases we hypothesize the occurrence of physical-chemical interactions between components of each of the extracts and AM in the gastrointestinal tract to explain these results. From the literature, a significant reduction of the absorption of AM induced by

orlistat in healthy volunteers was also observed (ZHI *et al.,* 2003); this drug, a lipase inhibitor, significantly reduced the systemic exposure to AM by approximately 25% and a decrease of similar magnitude (~25%) was detected in the generation of the metabolite MDEA (the major metabolite of AM). According to ZHI *et al.* (2003) the absorption of highly lipophilic drugs such as AM may depend on the presence of a lipid phase in the gastrointestinal environment, which may be affected by the pharmacological action of orlistat. The magnitude (C_{max}) and extent (AUC_{0-t}) of absorption of AM were also enhanced in healthy volunteers who received a single-dose of the drug immediately after consuming a high-fat meal *versus* following an overnight fast (MENG *et al.,* 2001). SHAYEGANPOUR, JUN and BROCKS (2005) also studied the effects of food on the pharmacokinetics of AM in rats. The results obtained concerning the interference of lipids on the oral bioavailability of AM corroborated those reported in humans.

Despite further studies are needed to understand the mechanism associated to the herb-drug interaction reported herein between *F. vesiculosus* extract and AM. It should be highlighted the overview recently published by COLALTO (2010) about herbal interactions on the absorption of drugs and the underlying interaction mechanisms. On the other hand, the dietary fibre may also reduce the drug absorption when both are assumed nearly by a mechanism of action similar to bile sequestration; herb-drug interactions at absorption level associated with dietary fibre have been reported, as instance, for lovastatin (RICHTER, JACOB and SCHWANDT, 1991), digoxin (BROWN and JUHL, 1976; BROWN, JUHL and WARNER, 1978), metformin (GIN, ORGERIE and AUBERTIN, 1989) and glibenclamide (NEUGEBAUER, AKPAN and ABSHAGEN, 1983). In addition, LODEIRO *et al.* (2012) recently reported that *F. vesiculosus* interacts with aluminum of acidic waters by a mechanism of adsorption, suggesting that these physicochemical data may be of interest in modeling drug-food interactions, particularly those referring to aluminum-containing antacids-food pharmacokinetic process produced in the gastrointestinal tract. Therefore, similar physicochemical adsorption mechanisms may occur between *F. vesiculosus* and AM.

In fact, for the first time were reported herb-drug interactions between *F. vesiculosus* extract and *P. cupana* extract with AM, which determined a considerable decrease on AM bioavailability in rats. Therefore, the therapeutic efficacy of AM may be compromised by the concurrent administration of herbal slimming medicines/dietary supplements containing *F. vesiculosus* or *P. cupana.*

On the other hand, it should be highlighted that the pre-treatment with *C. aurantium* extract significantly increased the peak concentration of AM, while the extent of systemic exposure was comparable between both groups. To explain this increase of the C_{max} in the group of rats pre-treated with *C. aurantium* we hypothesize that the extract or some of its constituents could change (increase) the gastrointestinal motility, thus increasing the rate at which AM passes to the intestine. Actually, the absorption of AM across the intestinal membrane occurs by passive diffusion, which is a non-saturable process (MARTÍN-ALGARRA *et al.,* 1997). According to the studies of JINZHAO *et al.* (2005), the *C. aurantium* may improve the gastric emptying in rats with functional dyspepsia. Furthermore, extracts of *C. aurantium* also increased the rate of gastrointestinal motility enhancing, therefore, the absorption function of gastrointestinal tract (LI *et al.,* 2007). In addition, *C. aurantium* can also antagonise the inhibition of the intestinal advance induced by atropine (XUE-BAO *et al.,* 2005). In a study conducted by FANG *et al.* (2009) the effects of constituents of *C. aurantium* on the gastrointestinal movement were evaluated; the hesperidin had a stimulatory effect on the gastrointestinal muscle contraction while synephrine had an inhibitory effect. Another possibility to explain the higher C_{max} values for AM in the group of rats pre-treated with *C. aurantium* extract could be related to a time-dependent inhibitory effects induced by *C. aurantium* or some of its phytochemicals on the cytochrome P450 (CYP) isoenzymes and/or P-glycoprotein (P-gp)–mediated efflux activity.

At last, in the rats pre-treated with *C. papaya* extract was observed a significant increase in the systemic exposure of AM (as assessed by AUC_{0-t} and $AUC_{0-\infty}$). *C. papaya* extract or some of its phytochemical constituents could have antimotility properties determining the inhibition of the intestinal propulsion movements (EZIKE *et al.,* 2009). This reduction of gastrointestinal motility can prolong the transit time of drugs and, consequently, it can increase the extent of the absorption of slightly soluble drugs, such as AM (TARIRAI, VILJOEN and HAMMAN, 2010). Another possibility to explain the higher AUC_{0-t} and $AUC_{0-\infty}$ values for AM in the group of rats pre-treated with *C. papaya* extract could be related to a time-dependent inhibitory effect on CYP isoenzymes and/or P-gp-mediated efflux activity.

In the pharmacokinetic studies performed were evaluated the effects of four herbal extracts separately, but also could be interesting to evaluate the effect of the combination of these herbal extracts in order to approximate to the commercially available products that normally are composed by a mixture of several components. These studies would also allow

realizing if the resulting effects could be additive, synergistic or antagonistic by the combination of the extracts. Taking into account our results, the combination of *F. vesiculosus* and *P. cupana* extract could probably result in a more significant decrease in the bioavailability of AM which could be quite problematic.

Overall, despite the safety and efficacy of *C. aurantium*-containing supplements have been frequently questioned, our results showed that the *C. aurantium* extract has minor effects on the pharmacokinetics of AM. These results reinforced that the *C. aurantium* extract should be maintained in the herbal dietary supplements.

Furthermore, taking into account the results obtained with *C. aurantium* extract and *C. papaya* extract *in vitro* studies to evaluate the impact of these extracts on the metabolism of AM should be also conducted using, for instance, a recent and promising *in vitro* hepatocellular model, the HepaRG cells.

The changes in body weight of the rats submitted to a 14-days treatment period with each of the extracts or the vehicle of the extract were not exactly the expected. The extracts of *F. vesiculosus*, *C. aurantium* and *C. papaya* were shown to be ineffective to control de body weight gain in rats during the period of 14 days. The only exception was *P. cupana* extract which was able to suppress gains in body weight of rats. Despite the evidence from the literature that these extracts have anti-obesity effects, our data do not support these findings, probably due to the short period of repeated administration of the extracts (only two weeks).

During the elaboration of this thesis, the European Medicines Agency (EMA) established an action Plan for Herbal Medicines 2010-2011 in which one of the objectives was to improve the output of the Committee on Herbal Medicinal Products, in particular by increasing the quality and number of Monographs and List entries. Effectively, the Committee on Herbal Medicinal Products (HMPC) of European Medicines Agency launched a call to encourage submission of scientific data on *P. cupana* (Guarana) in order to prepare the corresponding herbal monograph (EUROPEAN MEDICINES AGENCY, 2011b). The final document Monograph was already published and the information about drug interactions was included. This call for Monographs could be related to the widespread use of herbal products and probably to the increased risk of potential herb-drug interactions. In addition, this call for

Monographs demonstrates that the authorities have increasing interest in this field and with the work presented in this thesis we can give our contribute.

In the thesis we decide to evaluate the effects of the herbal *extracts of F. vesiculosus*, *P. cupana*, *C. aurantium* and *C. papaya* by the reasons explained previously but also could be interesting to evaluate *in vivo* the potential herb-drug interactions between AM and other commonly used weight loss herbal extracts such as *Garcinia cambogia, Hoodia gordonii* or *Ilex paraguariensis*. In addition, could also be interesting to evaluate the interactions between these extracts with other high-risk cardiovascular drugs of narrow therapeutc index such as warfarin and digoxin, which are frequentely involved in relevant herb-drug interactions.

In conclusion, despite the widespread use of herbal medicines, documented herb-drug interactions are sparse and many of the observed herb-drug interactions are based in *in vitro* studies. Although some herb-drug interactions may be beneficial by enhancing the efficacy and reducing the toxicities of the co-administered drugs, in most of the cases the herb-drug interactions may increase drug toxicity. Therefore, more studies are needed to confirm and assess the clinical significance of these potential herb-drug interactions. Our results provide an evidence of the effects of these extracts on the pharmacokinetics of AM in rats. Even so, to reliably assess the clinical effects of these standardized herbal extracts or of the commercially available products containing these extracts on the pharmacokinetics of AM clinical trials specifically designed to evaluate these potential herb-drug interactions are needed. These clinical trials would allow obtaining more information that could be included in the summary of product characteristics of AM and/or in Herbal Monographs.

VIII.2. BIBLIOGRAPHY

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CHAPTER IX

FINAL CONCLUSIONS

IX. FINAL CONCLUSIONS

IX.1. – CONCLUSIONS

The experimental work presented in this thesis consisted in the development and full validation of bioanalytical methods for the quantification of amiodarone (AM) and its major metabolite (mono-*N*-desethylamiodarone, MDEA). After that, a lot of studies were conducted in order to evaluate the potential for herb-drug pharmacokinetic interactions between various slimming herbal extracts and AM.

Briefly, the most relevant achievements and conclusions obtained throughout the work subjacent to the present thesis are the following:

- The development and full validation of the first high-performance liquid chromatography-diode array detection (HPLC-DAD) method for the simultaneous determination of AM and MDEA in human plasma using microextraction by packed sorbent (MEPS) was achieved. The MEPS procedure developed for plasma sample preparation has several advantages; it does not require the evaporation and reconstitution steps usually needed in most of the liquid-liquid extraction (LLE) or solid-phase extraction (SPE) techniques; MEPS presents a cost per analysis minimal compared to conventional SPE because each MEPS sorbent can be re-used several times before being discarded while SPE cartridges are indicated for single use only, and MEPS also enables the reduction of the organic solvent consumption and the sample preparation time.

- A new HPLC-DAD method using LLE was developed and fully validated in order to simultaneously quantify AM and MDEA in rat matrices (plasma and heart, liver, kidney and lung tissue homogenates). Its major advantages include the short chromatographic run time (only 5 min) using simple instrumentation and uncomplicated chromatographic conditions (isocratic elution and detection in UV spectral region); these aspects suggest that the method is appropriate for implementation in almost all laboratories, and fast bioanalytical assays are essential whenever a large number of samples have to be analysed.

- A significant decrease (55.4%) in the peak plasma concentration of AM was clearly demonstrated following the simultaneous co-administration of the *Fucus vesiculosus* extract and AM, as well as a reduction of approximately 30% in the extent of systemic drug exposure. On the other hand, no important effects were detected either on the rate or extent of systemic exposure to AM after the administration of the drug to pre-treated rats one day after the last treatment with *F. vesiculosus* extract. Hence, taking these findings together, it is apparent that *F. vesiculosus* extract or some of its components interact with AM in the gastrointestinal tract, reducing significantly the bioavailability of the drug; actually, this interaction was identified as relevant only after the simultaneous co-administration of *F. vesiculosus* extract and AM.

- The simultaneous co-administration of the *Paullinia cupana* extract and AM determined a significant decrease (73.2%) in the peak plasma concentration of the drug, as well as a reduction of approximately 60% in the extent of systemic drug exposure. A decrease in the tissue concentrations of AM and MDEA was also observed. On the other hand, no important effects were detected either on the rate or extent of systemic exposure to AM after the administration of the drug to pre-treated rats one day after the last treatment with *P. cupana* extract. Hence, taking these findings together, it is apparent that *P. cupana* extract or its components interact with AM in the gastrointestinal tract, reducing significantly the bioavailability of the drug.

- The simultaneous co-administration of the *Citrus aurantium* extract and AM has no important effects either on the magnitude or extent of systemic exposure to AM. However, in rats pre-treated with *C. aurantium* extract it should be highlighted the significant increase of the peak plasma concentration of AM, while the extent of systemic exposure was comparable between both groups. Overall, *C. aurantium* extract have only minor effects on the pharmacokinetics of AM in rats.

- The simultaneous co-administration of the *Carica papaya* extract and AM show also minor effects either on the magnitude or extent of systemic exposure to AM.

However, in the rats pre-treated with *C. papaya* extract it should be highlighted a marked increase in the extent of systemic exposure to AM (60-70%). Hence, taking these findings together, it is evident that *C. papaya* extract or its components interact with AM increasing significantly the bioavailability of the drug but this interaction was identified as relevant only after the continued administration of *C. papaya* extract.

In conclusion, the findings reported along this thesis showed the relevance of screening herb-drug pharmacokinetic interactions. The knowledge of relevant herb-drug interactions is certainly useful to help in the preparation of appropriate herbal monographs such those in course by the Herbal Medicinal Products Committee (HMPC) of the European Medicines Agency (EMA). The availability of this kind of information gathered together in a single and easily accessible document (monograph) may be of great value to promote a more rational and safer use of herbal products, having high importance in terms of protecting the public health.